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THE INTERACTION OF INFLUENZA VIRUS  
WITH NEUTRALIZING ANTIBODY

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## Summary

The polymeric antibodies secretory IgA (sIgA) and IgM are important in immunity to influenza virus and this work was undertaken primarily to investigate the mechanisms by which they neutralized type A influenza virus (A/fowl plague virus/Rostock/34; H7N1) (FPV/R). Secondly, this thesis presents data on the quantitative aspects of neutralization by monoclonal and polyclonal IgG, focusing particularly on the number of antibody molecules binding to virus particles at minimum and maximum levels of neutralization.

The attachment of FPV/R neutralized by sIgA and IgM was completely blocked at 4°C. The large molecular structure of these antibodies suggests that steric hindrance may be the mechanism by which attachment is impaired. However at 25 and 37°C neutralizing sIgA and IgM inhibited attachment of only 50% of the neutralized virus and none of this attached virus was internalized. It is inferred that IgM and sIgA interfere with the endocytotic event responsible for internalization of virus.

In contrast when neutralized by either IgG or monomeric IgA (obtained from the partial reduction of the above sIgA), FPV/R attached to and penetrated into EHK-21 cells, with the genome accumulating in the nucleus, at a rate indistinguishable from that of non-neutralized virus. This behaviour was independent of temperature from 4 to 37°C. Thus IgG and monomeric IgA neutralized infectivity at a stage subsequent to penetration. Virus was neutralized by F(ab')<sub>2</sub> and Fab' fragments of monoclonal anti-HA IgG. Neither prevented the attachment, penetration, or the accumulation of viral genomes in the nucleus.

Radiochemical data obtained on the binding of immunoglobulins (IgG, sIgA) to the haemagglutinin (HA) of FPV/R in suspension suggests that the virus is saturated at approximately one four-chain immunoglobulin unit per HA spike. Two-step competition assays show that the binding of one molecule of monoclonal IgG to a HA spike prevents the binding of monoclonal antibodies to two different epitopes on that spike and also the binding of polyclonal antibody to the HA. These observations argue against direct epitope-epitope competition and for a steric or allosteric block on the binding of further IgG to the HA spike by prebound IgG.

The kinetics of neutralization of FPV/R at 4°C with minimal concentrations of monoclonal IgG suggested that 3 IgG molecules were required to neutralize an infectious virus particle. The discrepancy between this and radiochemical data indicating that least 50 antibodies per virus particle are required for neutralization is reconciled by a theory that neutralization only occurs when antibody binds to certain "neutralization relevant" HA spikes, which are in the minority, and differ from "neutralization irrelevant" spikes only in their interaction with the viral core.

To Petra



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## ABBREVIATIONS

All abbreviations are defined at first mention in text. The following list defines key abbreviations used in this thesis.

BHK	Baby hamster kidney
BrHA	bromelain released HA
BSA	Bovine serum albumin
CEF	Chick embryo fibroblast
CRU	Cellular receptor unit
DNA	Deoxyribonucleic acid
Fab'	antigen binding fragment
F(ab') <sub>2</sub>	Divalent antigen binding fragment
Fc	crystalizable fragment
FcR	Fc Receptor
FPV/D	Influenza A/fowl plague/Dutch/27 (H7N7)
FPV/R	Influenza A/fowl plague/Rostock/34 (H7N1)
HA	Haemagglutinin
HAU	Haemagglutination units
HEF	Human foreskin fibroblast
HI	Haemagglutination inhibition
HIU	Haemagglutination inhibition units
IgA	Immunoglobulin A (monomeric)
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MW	Molecular weight (also Mol. wt.)
NA	Neuraminidase
N-Ag	Neutralization antigenic site
NCS	Newborn calf serum
N-Ep	Neutralization epitope

NI	NA inhibition
NP	Nucleocapsid protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PFU	Plaque forming unit
PP	Peyer's patches
RAM	Rabbit anti-mouse
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
Sc	Secretory component
SDS	Sodium dodecyl sulphate
SFV	Semliki Forest virus
sIgA	Secretory IgA
TBS	Tris buffered saline
V31	Reassortant influenza A/V31/83 (H7N2)
VAP	Virus attachment protein
VAS	Virus attachment site
w/v	weight:volume

**Declaration**

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work represented was done by myself, with the exception of those instances where the contributions of others has been acknowledged. All sources of information have been specifically acknowledged by reference.

A handwritten signature in cursive script, reading "Howard Taylor". The signature is written in dark ink and is positioned above the printed name.

Howard P. Taylor



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This work was conceived with a great deal of help and support from my supervisor Professor Nigel Dimmock. I am grateful to him for his advice throughout its progress.

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GENERAL INTRODUCTION

## INFLUENZA VIRUS

### 1. An overview

#### 1.1. Classification of strains

Three antigenically distinct types of human influenza virus A, B and C have been identified according to the cross reactivity of their major internal protein, the nucleoprotein (Smith et al., 1933; Francis, 1940; Taylor, 1949). Type A is the most most extensively studied influenza virus strain and is the most important epidemiologically and pathologically. Influenza A viruses cause epidemics in birds, swine, horses and seals as well as man. Whereas influenza B is thought only to infect humans, studies have recently shown that influenza C can infect pigs as well as humans (Guo et al., 1983). The three types share many common molecular and structural features (Webster et al., 1982; Scholtissek, 1983), however influenza C can be distinguished from types A and B in that it does not possess the surface glycoprotein neuraminidase. The influenza C virus is able to free itself from receptors (section 2.4) by the action of neuraminate-O-acetylerase (Herrler et al., 1985)

While many distinct subtypes of type A have been recognized, none are known for types B and C. Within the type A viruses, a further classification is based on the immunological

cross-reactivity of the haemagglutinin (HA) and neuraminidase (NA) surface antigens. Among type A viruses 13 HA subtypes (designated H1 to H13) and 9 NA subtypes (N1-N9) have been distinguished so far (WHO memorandum, 1980). Three influenza A combinations, with the antigenic compositions of: H1N1 (1918-1957, 1977-present), H2N2 (1957-1968) and H3N2 (1968-present) have been responsible for influenza epidemics in man this century. The generation of completely new subtype antigens is called antigenic shift and the initial appearance of a new subtype has resulted in major pandemics since the population has no immunity to the new antigens. In the interim periods between antigenic shifts influenza A undergoes minor antigenic variation or drift causing epidemics. This means that included among influenza A viruses of a common subtype designation there will be strains showing considerable antigenic variation. Thus the human influenza virus A/Bangkok/1/79 (H3N2) contains HA and NA antigens demonstrably related to those of influenza virus A/Hong Kong/68 (H3N2) but show degrees of differences from them.

The nomenclature system can be understood as follows: A/fowl plague/Rostock/34 (H7N1) refers to an influenza A virus isolated from domestic poultry in Rostock (Germany, now the German Democratic Republic) in 1934 with an HA of subtype H7 and a NA of the subtype N1. This avian influenza virus was used in the majority of studies described in this thesis and the following review of the features of influenza and immunity to influenza

will concentrate on the studies of type A influenza viruses.

### 1.2. Structure of the influenza virus particle

Influenza viruses are best described as being highly pleiomorphic enveloped viruses. Examination of particles of influenza virus by electron microscopy has shown that there is considerable variation in particle shape and size which is dependent on strain and isolation (Horne et al., 1960; Bachi et al., 1969; Tiffany and Blough, 1970 a,b; Wrigley, 1979). Influenza particles are spherical or filamentous. Particles cultured in the chlorioallantoic membrane of chick embryos have a spherical or roughly spherical shape typically 80-120 nm in diameter (Tiffany and Blough, 1970a; Nermut and Frank, 1971; Wrigley, 1979). The percentage exhibiting spherical morphology increases with passage (Choppin et al., 1960). Fresh isolates from man and other species show a much greater shape variation including elongated filamentous particles of over 1u in length (Wrigley, 1979). These probably represent the true state of influenza particles in nature.

The virus particle has a lipid envelope that is derived from the host cell (Kates et al., 1961; Klenk and Choppin, 1969; 1970) and this is studded with two types of morphologically distinguishable glycoprotein spikes which project radially outwards from all over the virus surface. The phospholipid,

glycolipid and cholesterol composition of the virus membrane is a reflection qualitatively and quantitatively of that of the host membrane from which the virus was formed during budding (Patzner et al., 1979). The HA spike is roughly triangular in cross section and extends approximately 12 nm from the lipid membrane (Laver and Valentine, 1969) whereas the NA takes on the form of a mushroom but with a narrow stalk (Wrigley et al., 1973). The HA protein is the influenza virus attachment protein (VAP) interacting with terminal neuraminic acid cellular receptor units (CRUs) that are contained on plasma membrane glycoproteins or glycolipids. The HA is also involved in the penetration of the virus into the cell. The NA protein cleaves neuraminic acid from receptors on the cell surface as well as from other sialoglycoproteins and glycolipids. Both HA and NA have now been crystallized and their three-dimensional structures established by X-ray crystallography (Wilson et al., 1981; Wiley et al., 1981; Varghese et al., 1983; Colman et al., 1983). The conspicuous spikes are quoted as numbering between 700 and 1000 per virion (Tiffany and Blough, 1970b; Wrigley, 1979) in a proportion of roughly 5 HA spikes to 1 NA, but again these statistics are dependent on the strain of virus. The spikes are closely packed in an even manner, each spike having six neighbouring spikes on average according to the principal of the honeycomb (Wrigley, 1979). It is now thought that the NA spikes are clustered in discrete areas on the virion while the HA spikes are distributed uniformly over the virus surface (Murti and Webster, 1986). Both spikes are thought to penetrate through the

lipid bilayer via hydrophobic amino acids and may make contact with the underlying electro-dense layer of matrix (M1) protein.

Encompassed by the lipid bilayer is a 6nm thick shell or matrix protein which is composed of the M1 protein. Within this are the ribonucleoprotein (RNP) structures consisting of 4 protein components and the virus genome of 8 molecular species of single stranded RNAs of differing coding potential (Inglis et al., 1976). The predominant protein is the nucleoprotein (NP) and in smaller quantities are the polymerase proteins PB1, PB2 and PA. The 3 polymerase proteins constitute an RNA transcriptase enzyme (Kawakami and Ishihama, 1983). In the RNP complex the subunits of NP are arranged like beads on a string coiled doubled back on itself and then twisted into a helix (Compans et al., 1972). It has been calculated that approximately 20 nucleotides of RNA interact with a single NP subunit (Winter and Fields, 1981). The RNA is partially exposed since it is susceptible to RNase activity. Since RNase activity also destroys the RNP, RNA must be an integral part of the total structure.

### 1.3. Genome structure

The genetic information of influenza A viruses resides in eight segments of single stranded RNA. These code for 7 virion structural proteins (PB1, PB2, PA, HA, NA, NP and M<sub>1</sub>) and three nonstructural proteins found only in infected cells (NS<sub>1</sub>, NS<sub>2</sub> and

M<sub>2</sub>). The virus RNA is negatively stranded, i.e. viral mRNAs are complementary to genome RNAs. Naked virion RNA is not infectious because it must be transcribed by a virion associated RNA-dependent RNA transcriptase.

The segmented genome also allows a high degree of genetic recombination between influenza A strains. This reassortment plays an important part in the initiation of influenza epidemics throughout the world since it can result in expression on the virion surface of new HA and NA antigens (antigenic shift) against which the population has no immunity.

The demonstration that the eight RNAs are distinct was shown by two dimensional oligonucleotide fingerprinting (McGeoch et al., 1976). The assignments of viral polypeptides to RNA segments have been made (Review: Lamb and Choppin, 1983). The gene assignments are indicated in the discussion of the influenza virus proteins in section 2.



## 2. Influenza protein structure and function

The biological characteristics of the influenza virus are intrinsic to the nature, composition and function of its viral proteins. Reviewed below are more detailed descriptions of the structure and function of the proteins of influenza.

### 2.1. Transcriptase associated proteins; PB1, PB2, and PA (encoded by RNA segments 1,2 and 3 respectively).

The three largest RNA segments direct the synthesis of single proteins MW 82,000 to 95,000. PB1, PB2 and PA (Horisberger, 1980) are components of the transcriptase complex in viruses and infected cells (Bishop et al., 1972; Bean and Simpson, 1973). These proteins are present in very small amounts in the virion (less than 3%). It is known that PB1 and PB2 are required for mRNA synthesis and PA possibly for vRNA synthesis (Scholtissek and Bowles, 1975; Palese et al., 1977). In investigating *in vitro* transcriptase reactions it has been found that PB2 is involved in binding to the cap structure of host cell mRNA (Ulmanen et al., 1981; Penn et al., 1982) and that PB1 initiates transcription (Ulmanen et al., 1983). Braam et al (1983) showed that a complex of PB1, PB2, and PA proteins move down the elongating viral mRNA chains constituting a transcriptase catalyzing mRNA synthesis.

## 2.2. Haemagglutinin (HA) (encoded by RNA segment 4).

The HA glycoproteins of influenza virus membranes are responsible for the attachment of influenza to cells by interacting with the sialic acid residues on cellular receptor molecules (section 3.1). In fact, HA is a multifunctional protein since as well as mediating attachment (Lazarowitz and Choppin, 1975) and the agglutination of erythrocytes (Hirst, 1941), it is a major determinant of virulence of influenza (Bosch et al., 1979; Rott, 1979), the most important viral antigen to which neutralizing antibodies are directed (Laver and Kilbourne, 1966; Virelizier, 1975) and has been strongly implicated in mediating fusion events (White et al., 1982; Wharton et al., 1986) which result in the initiation of the replication of influenza in the cell (section 3.3). Amino acid sequence variation resulting in altered antigenic properties of the HA accompany the recurrent epidemics of influenza respiratory disease in man. This variation can occur in two ways. Small changes in antigenicity (antigenic drift) are the result of a gradual accumulation of point mutations, while the complete change in antigenic properties (antigenic shift) involves the replacement of the gene coding for one HA with that for another (section 5.2).

In 1981 Wilson and his colleagues using X-ray crystallography described the three dimensional structure of the HA of a H3N2 influenza virus. This together with data on its functions means that the HA is now one of the most extensively

characterized of all protein molecules. For instance we know the complete amino acid sequences of several HAs (Porter et al., 1979; Gething et al., 1980; Palese and Young, 1982), that the HA has a trimeric structure (Wiley et al., 1981; Wilson et al., 1981), its orientation with respect to the membrane (Skehel and Waterfield, 1975), the location of the neutralizing antigenic sites (Wiley et al., 1981; Caton et al., 1982), the glycosylation sites (Gething et al., 1980; Ward and Dolpheid, 1981; Wilson et al., 1981), the probable location of the cell binding site (Wilson et al., 1981; Rogers et al., 1983) and fusion sequences (Richardson et al., 1980; Wilson et al., 1981; White et al., 1982).

The determination to 0.3nm resolution of the three-dimensional structure of the A/Hong Kong/1968 HA (H3) has revealed that the HA spike is a non-covalently linked trimer (Wiley et al., 1981). The structure of the H3 is a good model for other HAs since sequence comparisons of other influenza HAs show that many structurally important residues are conserved.

The HA monomer is synthesized as a single polypeptide which undergoes post-translational cleavage in 3 places. The synthetic pathway follows the route typical of membrane glycoproteins. After translation of the HA monomer the N-terminal signal sequence is removed by proteolysis (Air, 1979; Waterfield et al., 1979) and the monomer is moved from the endoplasmic reticulum to the Golgi apparatus. Whilst in the endoplasmic reticulum the

elongating chain is glycosylated, oligosaccharides are added to the polypeptide (Klenk et al., 1974), these are then further modified in the Golgi apparatus where the addition of N-acetyl glycosamine, galactose, terminal sugars and sialic acid occurs (Compans, 1973b; Rothman and Lodish, 1977; Elder et al., 1979). Also in the Golgi the glycoprotein becomes acylated (Schmidt, 1982).

Depending on the host cell and virus strain, the HA molecule is cleaved with the removal of one or more intervening residues to give two polypeptide chains; HA1 (36,000 daltons MW) and HA2 (27,000 daltons MW). This proteolytic cleavage is required for activating infectivity but does not affect antigenic or receptor binding properties. The HA1 and HA2 remain joined by a single disulphide bond. In addition to this inter-peptide bond there are four disulphide bonds within the HA1 and one in the HA2. Three HA1 and HA2 dimers come together to form a trimeric HA. It is likely that a coated vesicle transports the proteins to the plasma membrane. Further glycosylation modifications occur during the transport of the vesicle to the plasma membrane (Compans, 1973b; Rott and Klenk, 1977). What results is a typical integral membrane glycoprotein with a three domain structure. Each monomer is glycosylated at 5-7 sites so that the total carbohydrate content is approximately 19% w/w.

Following fusion of the coated vesicle with the plasma membrane a large hydrophilic carbohydrate-containing N-terminal

domain becomes the external portion of the polypeptide, whilst a small uncharged hydrophobic peptide of 25-28 amino acids spans the membrane at the C terminus of the HA. A smaller hydrophilic domain at the C-terminus about 10 amino acids long lies on the internal side of the membrane. Gething and Sambrook (1981) found that removal of the portion of the HA gene that codes for the anchor sequence results in the HA glycoprotein being secreted from the cell. Thus when the mature virion buds from the cell (section 3.6) the HA will be firmly anchored in, and penetrate through, the viral membrane (Figure 2.1.1).

The HA monomers within the HA spike form a cylinder 13nm long consisting of alpha triple stranded coiled-coil of a helices extending 7.6nm from the membrane and then forming into a distal globular region of anti-parallel beta sheets (Wilson et al., 1981) (Figure 2.1.1). The receptor binding site and the variable antibody binding regions are located on the surface of this globular region which is composed entirely of HA1 sequences (Wiley et al., 1981). Each monomer has a loop-like topography; it begins at the membrane, extends 13.5nm distally then folds back to enter the membrane via the N-terminus of HA1. The C-terminus of HA1 is 2.1nm from the N-terminus of HA2, indicating a substantial rearrangement in this region when HA is activated by cleavage.

A highly conserved region in a surface pocket on the distal end of the monomer has been implicated as the cell-receptor binding site (Wiley et al., 1981). Rogers et al. (1983) have

observed modifications of sialic acid binding specificity as a direct consequence of amino acid substitution at a residue (226) which lies in the proposed receptor pocket.

Four neutralization antigenic (N-Ag) sites have been identified by the analysis of the amino acid sequence of HAs of naturally occurring H3 variants (Wiley et al., 1981) and of antigenic escape mutants selected by monoclonal antibodies (Yewdell et al., 1979). The N-Ag sites have been labelled A,B,C, and D (Figures 2.1.1; 2.1.2).

A N-Ag site is a region of protein that induces and binds neutralizing antibodies. Within N-Ag sites there are neutralizing epitopes (N-Ep) which are the individual sequences of amino acids that a neutralizing monoclonal antibody will bind to. A N-Ag site may therefore express many N-Eps. Knossow et al. (1984) found that the simple addition of an amino acid side chain in the HA results in only minor local distortions of the HA and that this is a sufficient structural alteration for a virus to escape neutralization. Proposals (Webster and Laver, 1980) of large conformational changes to account for variation in HA antigenicity are unnecessary in this and perhaps other cases.

Site A is a "loop" that protrudes out from the monomer surface by 0.8 nm containing residues 133-137 and 140-146. Site B, the "tip", comprises the external residues, 155-160 and 186-197, of an alpha helix which lie near to (but do not encroach

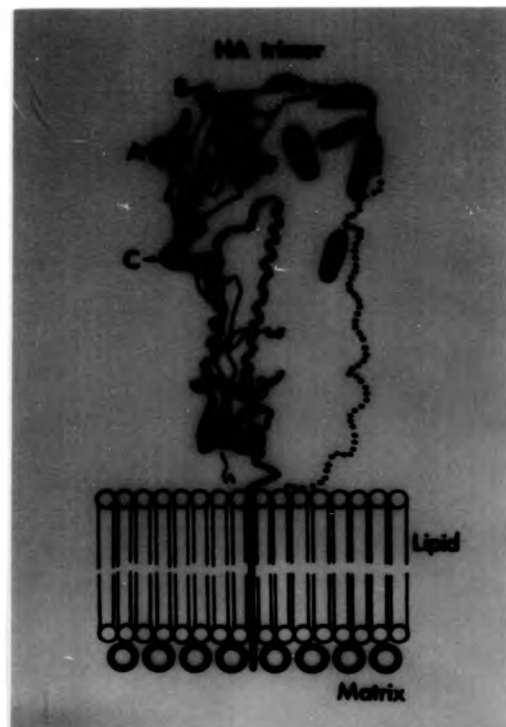


Figure 2.1.1. Diagrammatic representation of influenza virus HA (H3) trimer.

A carbon chain representation of the polypeptide (Wilson et al., 1981) is shown for a single HA subunit within the trimer. The HA2 polypeptide is designated in a thicker line whilst HA1 is shown in a thin line. The receptor sites (in red) and the antibody binding regions\* (in green, designated A-D on the highlighted HA subunit) are shown. Each H3 HA trimer has 12 antibody binding regions and 3 receptor sites. The outlines of the two other HA monomers are marked by the dotted line. The HA trimer is inserted into the viral lipid membrane. The N-terminus of HA2 has fusion activity whilst the C-terminus of the HA2 penetrates through the lipid layer to come in approximate contact with the M monolayer.

\* There are five antibody binding regions on H1 (Caton et al., 1982).

Figure 2.1.2. Diagrammatic representation of the antigenic regions on the H3 HA molecule.

Orientated as in Figure 2.1.1 with end inserted into membrane at the bottom and the sialic acid binding site at the top. The four antibody binding sites determined by sequence analysis of natural variants and escape mutants selected with monoclonal antibodies are shown in green. Two of the regions recognized by human Th cell clones 57 and 58 are shown as red lines. Adapted from Wiley et al. (1981) and Mitchell et al. (1985).

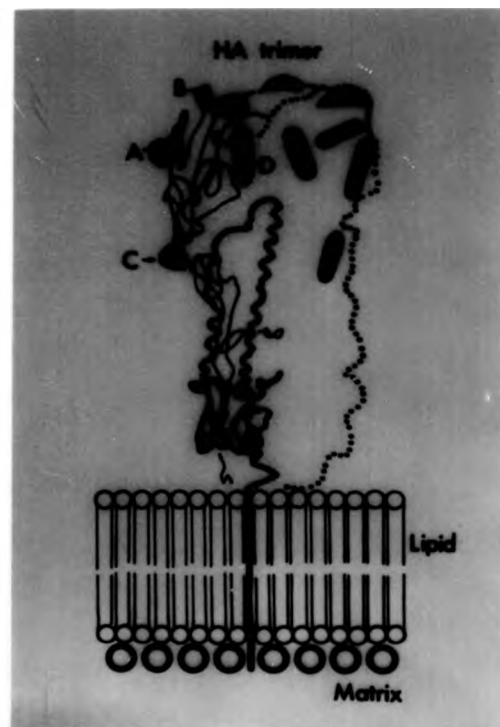


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on) the pocket implicated in VAP-CRU interaction. Site C is located on a "bulge" on the tertiary structure. This is at the disulphide bond between cysteine residues 52 and 277 and is located 6nm from the top of the molecule. In site D amino acid substitutions occur at a monomer-monomer interface. It is possible that this area contains antigenic determinants which are composed of residues from two or three of the subunits (Nestorowicz et al., 1985) and that antibodies recognize quarternary epitopes. Site A is considered to be immunodominant as judged by the epidemiological significance of viruses expressing altered antigenicity at this site and by the predominance of monoclonal antibodies binding to this site (Webster and Laver, 1980). An operational antigenic map of the H1 subtype of influenza A/PR/8/34 by Caton et al. (1982) defines the presence of five antigenic sites. Two of the sites on H1 correspond to sites A and D on H3 but the locations of the others do not correspond to those on H3. Antigenic sites that corresponded directly contained no potential glycosylation sites (Asn-X-Ser/Thr), but such a sequence does occur in a region of the H3 corresponding to antigenic site Cb on H1, and in the H1 at the region corresponding to the H3 antigenic site C. The reciprocal nature of this relationship where the antigenic sites on the HA of one subtype do not contain glycosylation sites that are found on the equivalent location of the HA of the other subtype suggested that carbohydrate might be modulating the antigenic structure of the HA by masking regions of the protein from recognition by antibody (oligosaccharide chains are host

specific and presumably not very immunogenic in another infected animal of the same species). Direct evidence was presented by Skehel et al. (1984) who showed that the substitution of a glycosylation site at residue 63 in the HA of the 1968 Hong Kong (H3) influenza blocked the recognition by a monoclonal antibody directed to the site near that residue. Therefore the localization of carbohydrate on HA is an important consideration as antigenicity can be affected. Carbohydrate may also cover proteolytic cleavage sites.

In addition to antibody binding sites on HA there are determinants that are recognized by helper T (Th) cells. Th cells play a crucial part in the generation of both antibody (B cell) and cytotoxic T cell immune responses (section 5). Characterization studies of the human Th response (Green et al., 1982) and the murine Th response (Hurwitz et al., 1984) have shown that influenza strain specific T cells recognize determinants different from those seen by anti-HA antibodies (Figure 2.1.2). Using a T cell proliferation assay Katz et al (1985) found that T cells from mice primed with one strain of influenza virus cross-reacted with another HA of the same subtype and serologically distinct subtypes. The major components in the T cell response to both homologous and heterologous HA was the recognition of determinants within the HA2 polypeptide which is not a feature of the B cell response.

### 2.3. Nucleocapsid protein (NP) (encoded by RNA segment 5).

The nucleoprotein (NP) is an internal non-glycosylated protein (60,000 daltons MW) and is one of the group specific antigens of influenza viruses that distinguishes between influenza A, B and C. It comprises up to 25% of the total protein of the virion (Skehel and Schild, 1971). NP interacts with itself and is intimately associated with the PB1, PB2 and PA proteins. RNP (consisting of the polymerase proteins, NP and the 8 RNAs) has also been found on the basis of biochemical evidence to interact with matrix protein (Rees and Dimmock, 1981). NP undergoes antigenic variation and possesses at least 3 non-overlapping antigenic sites, one being common to all strains tested (Van Wyke et al., 1980). Monoclonal antibody to this conserved domain inhibited *in vitro* transcription of viral RNA, suggesting that NP is involved in RNA transcription. Based on the immunological evidence that T cells recognize NP (Townsend et al., 1984) and NP is detected at the surface of infected cells by antibody (Virelizier et al., 1977; Lamb et al., 1982) it is suggested that NP is expressed on the surface of cells.

#### 2.4. Neuraminidase (NA) (encoded by RNA segment 6).

The other integral membrane glycoprotein that protrudes from the surface of the influenza virus is neuraminidase (NA). It is a tetramer of molecular weight 240,000, reducing to 200,000 when solubilized by pronase (Blok et al., 1982). The mushroom shaped spikes of NA are present in roughly one fifth the amount of HA spikes and constitute about 5% of the protein of the virion (Skehel and Schild, 1971). Chemically neuraminidase hydrolytically cleaves the glycosidic bond joining the keto group of N-acetyl neuraminic acid (sialic acid) to D-galactose or D-galactosamine (Gottschalk, 1957).

When Varghese et al. (1983) described the detailed structure of the NA at 0.29 nm using X-ray crystallography they provided the first known example of a tetrameric protein with 4 fold symmetry. The polypeptide chain folds into six topologically identical, four stranded antiparallel beta sheets which are themselves arranged like the blades of a propeller. The tetramer can be visualized as an oligomer of identical polypeptide chains with a box-shaped globular head connected to the virus membrane by a long slender stalk, giving the well documented mushroom shape of the spike (Laver and Valentine 1969; Wrigley et al., 1973). The three dimensional structure of the stem is unknown at present, but evidence suggests that it is a relatively unfolded extended polypeptide and may be flexible (Wiley, 1983). It is known that amino acids 1-6 are on the cytoplasmic side of the membrane followed by an uncharged and primarily hydrophobic peptide (7-35) which spans the membrane and anchors the NA to the lipid. The N-terminal location of the membrane anchor peptide is

an uncommon feature for a glycoprotein, the HA and most other viral glycoproteins having C-terminal anchoring hydrophobic sequences. Approximately half of the carbohydrate associated with the neuraminidase of influenza B/Lee/40 is located on the stalk (Allen et al., 1977).

The biological role of neuraminidase activity is unclear but appears to confer on influenza virus the ability to free itself from neuraminic acid containing structures. This may promote the elution of progeny virus from host-cell surface (Palese and Schulman, 1974) during virus assembly. The removal of sialic acid from the carbohydrate moieties of newly synthesized HA and NA is necessary to prevent self aggregation of virions (Palese et al., 1974). Removal of sialic acid by NA may also expose HA to cleavage (Schulman and Palese, 1977). Studies on fusion of cell membranes with liposomes containing influenza glycoproteins and observations that the fusion of erythrocytes was prevented by anti NA antibodies, (Huang et al., 1980; 1985) suggest a requirement of NA for membrane fusion (section 3.3). This however is difficult to reconcile with fusion of CV-1 cells expressing cloned HA gene (White et al., 1982). The respiratory tract is lined with sialomucoproteins which bind the influenza virus and therefore decrease infectivity (Shen and Ginsberg, 1968). NA may have a role here in enabling the virus to be freed from these inhibitory proteins and allowing it to reach target epithelial cells. In general then the enzyme catalytic properties of influenza neuraminidase facilitates the "mobility" of the virus both to and from the site of infection. The biological

significance of the apparent distribution of NA in discrete areas on the influenza particle (Murti and Webster, 1986) is not understood, but the clustered arrangement of NA may be important in the optimum performance of NA functions.

Antibodies to NA are not neutralizing (Webster and Laver, 1967; Kilbourne et al., 1968) but by aggregating virus at the cell surface can prevent viral budding and restrict multiple cycles of replication in susceptible cells (Webster and Laver, 1967) and in mice (Schulman et al., 1968). Antigenic variants have been selected with monoclonal antibodies to neuraminidase (Webster et al., 1982). Therefore anti-NA antibodies can exert selective pressure on influenza and so have a role in the epidemiology of the disease. Colman et al. (1983) were able to propose the location of antigenic determinants on the NA molecule by examining the position of amino acid substitutions in the NA of antigenic escape mutants selected by monoclonal antibodies and the changes which occur in NA-antigenic drift naturally. The antigenic sites form a nearly continuous surface that encircle the catalytic site on the globular head of the molecule. The active site itself is conserved from strain to strain and is thought to be inaccessible to antibody, since inhibition of enzyme activity by monoclonal antibodies is only seen when large substrates such as fetuin are employed, not with small substrates such as N-acetylneuraminyllactose (Jackson and Webster, 1982), which implies an indirect steric effect of antibody. The inaccessibility of the catalytic site to antibody also suggests

that antibodies cannot exert pressure on that site for its modification.

#### 2.5. Matrix protein (M<sub>1</sub>) and M<sub>2</sub> (encoded by RNA segment 7).

Matrix or membrane (M<sub>1</sub>) protein is the most abundant virion protein composing up to 35% of the total virion (Skehel and Schild, 1971) and is thought to form a protein shell beneath the lipid bilayer. This hydrophobic protein interacts strongly with viral lipid and phosphatidylcholine vesicles *in vitro* (Gregoriades, 1980) and this would agree with the interpretation of Lenard et al. (1974), that M may be interacting with the lipid bilayer in the virus particle. Association of M with the lipid could also contribute towards the stabilization of the virus bilayer. As well as being a structural protein M<sub>1</sub> may recognize the viral glycoproteins and form a domain on the inner surface of the plasma membrane that subsequently provides a binding site for nucleoprotein during virion assembly. Little is known about interactions between spike glycoproteins, the M proteins and nucleocapsid proteins. However, based on immunological and protease accessibility experiments (Ada and Yap, 1979; Reginster et al., 1979) it is suggested that the M protein is exposed on the external surface of cells.

M<sub>1</sub> is type specific, being antigenically similar for all influenza A viruses tested (Schild, 1972). However, monoclonal

antibodies can discriminate small antigenic differences between subtypes (Van Wyke et al., 1980; Le Comte and Oxford, 1981).

Segment 7 also codes for the protein M2 (15,000 daltons MW). Lamb et al. (1985) showed that M2 is a glycoprotein which occurs in the infected cell and is inserted into the plasma membrane with its N-terminus external. Despite this membrane involvement it has not been detected in virus particles (Zebedee et al., 1985). It was demonstrated that anti-peptide antibodies could recognize the external M2 protein, and it was suggested that the protein could be an antigen in natural infection, however there is presently no direct evidence to support this idea (section 5.4). Hay et al. (1985) examined the genetics of sensitivity of influenza virus strains to amantadine, which acts at an early stage of infection, possibly virus uncoating. It was concluded that M2 was the primary determinant of amantadine sensitivity. While this supports the view that M2 is present in the virion and has a functional role, Zebedee et al. (1985) were not able to detect M2 in virus particles. Another protein, M3, has been predicted to be coded by segment 7 by sequence alone (Lamb et al., 1981) and this, as yet unidentified peptide may also be synthesized in infected cells.

#### 2.6. Nonstructural proteins NS<sub>1</sub> and NS<sub>2</sub> (encoded by RNA segment 8).

Two nonstructural proteins NS<sub>1</sub> (26,000 daltons MW) and NS<sub>2</sub>



(14,000 daltons MW) are coded by the smallest RNA segment. NS<sub>1</sub> is made in large amounts in infected cells (Compans et al., 1970), is phosphorylated (Almond and Felsenreich, 1982) and has been found associated with polysomes and in the nucleolus (Dimmock, 1969; Lazarowitz et al., 1973; Compans, 1973a; Shaw and Compans, 1978) Late in infection NS<sub>1</sub> of some but not all influenza virus strains form electron dense crystalline arrays with cellular RNA species (Shaw and Compans, 1978; Yoshida et al., 1981; Petri and Dimmock, 1981).

The functions of NS<sub>1</sub> and NS<sub>2</sub> have not been fully established but it has been suggested that NS<sub>1</sub> is involved in the shut-off of host cell protein synthesis and in the synthesis of virion RNA (Lazarowitz et al., 1973; Compans, 1973a; Wolstenholme et al., 1980).

### 3. Early influenza virus-cell interactions leading to virus infection.

In order to infect a cell the virus genome of an enveloped virus such as influenza virus has to cross both the cell plasma membrane and its own viral envelope. The question of how viruses enter cells remains controversial (Reviews: Dimmock, 1982; Marsh, 1984). For influenza virus the initial stages of infection appear to involve the binding of an influenza virus particle to a sialic acid containing receptor at the cell surface, absorptive endocytosis (viropexis), endosome formation followed by fusion of the influenza virus membrane with the membrane of the endosomes (lysosomal vesicle). An alternative route of entry suggests attachment is followed directly by fusion between the virus envelope and the plasma membrane leading to release of viral RNA; thus penetration and uncoating would occur in a single step.

#### 3.1. Attachment.

To firmly attach to a cell influenza virus binds via the HA to N-acetyl neuraminic acid (sialic acid) residues on oligosaccharide side chains of cellular glycolipids (gangliosides) or glycoproteins. Sialic acids are also essential CRUs for paramyxoviruses, polyoma viruses and encephalomyocarditis viruses. These viruses can exhibit strict and varied specificities for the sialyoligosaccharide linkages. The terminal sialic acid alone determines a glycoprotein's ability to act as a CRU for influenza virus (Suttajit and

Winzler, 1971, Huang, 1973). Closely related strains of influenza virus and escape mutants selected by monoclonal antibodies can differ in their specificity for sialyl linkages (Carroll et al., 1981; Rogers et al., 1983).

Glycophorin A is the main sialoglycoprotein molecule carrying CRUs for influenza virus on erythrocytes (Jackson et al., 1973). As a result of the use of haemagglutination as a quantification of virus titre and inhibition of haemagglutination as a measure of antibody titre, glycophorin A has become the "model receptor" for influenza virus. Inhibition of haemagglutination, however, does not necessarily correlate to neutralization, as it has been found that neutralized virus binds normally to cultured cells (Possee and Dimmock, 1981; Dimmock, 1984). Also it has been found that antibody to some avian and seal influenza viruses fail to inhibit haemagglutination of the virus yet effectively neutralize infectivity (Yoden et al., 1982; Kida et al., 1982). Therefore erythrocytes with short glycophorins reaching 5nm from the erythrocyte surface (Viitala and Jarnefelt, 1985) may be a poor model for the infectious process. Further weight is added to this view when it is considered that the "hedgehog-like" glycophorins lie deep amongst the much taller (10-15nm) poly N-acetyllactosamine chains which in turn are immersed in a "web" of carbohydrate molecules. The amount of carbohydrate covering the surface of erythrocytes is considerable compared to that of other cells (Viitala and Jarnefelt, 1985) and may have consequences for the attachment of

viruses. Other molecules carrying CRUs on the wide range of cells to which influenza virus binds to have yet to be characterized.

Attachment of influenza virus to cells, as with some other viruses, occurs at low temperatures (4°C) as well as at physiological temperatures (Kato and Eggers, 1969; Dourmashkin and Tyrrell, 1970; Stephenson and Dimmock, 1975). At physiological pH both virus and cells are negatively charged and tend to repel one another, if the pH is altered the charge will be affected and therefore this will have an impact on attachment. Huang (1974) found that charged agarose beads could adsorb influenza virus and the adsorption of influenza virus to neuraminidase-treated erythrocytes was restored when cells were treated with buffers at pH 3, 4 and 9. These results indicated that when a suitable ionic status of the cell surface is achieved influenza virus can bind in the absence of sialic acid. Physiological differences in pH may have a role in host-virus interactions, e.g. in the infection of intestinal tracts of birds with avian influenza viruses (Webster et al., 1978). Preferentially virus attaches to certain recognizable morphological features such as microvilli (Bachi et al., 1969; Helenius et al., 1980; Matlin et al., 1981).

DEAE-dextran enhances virus infectivity by increasing binding and underscores the lack of requirement for specific

surface molecules as receptors (Lenard and Miller, 1983). DEAE-dextran increased BHK cell associated vesicular stomatitis virus (VSV) particles by 3-4 fold. Since both VSV envelopes and cell surfaces are negatively charged at neutral pH the DEAE-dextran mediated binding is probably electrostatic.

A reflection of the ability of neuraminidase to hydrolyze sialic acid is that the attachment of influenza virus to a cell may be reversible. The attachment of influenza virions bound to erythrocytes at 4°C may be reversed by increasing the temperature to 37°C (Laver and Kilbourne, 1966). The reversal of attachment in this example is non-physiological as it is only seen when virus is in vast excess.

### 3.2. Internalization of influenza virus by the cell.

Paramyxoviruses can penetrate the cell surface by fusion, thereby achieving penetration and uncoating in a single step (Reviews: Choppin and Compans, 1975; Poste and Pasternak, 1978). The envelopes of these viruses fuse with the host cell membrane at pH7. Fusion in paramyxoviruses is mediated by the F or fusion glycoprotein present on the external surface of the viral envelope. While it is clear that paramyxoviruses can fuse at the cell surface, it is not clear that that this route is infective. There is no direct evidence that influenza A viruses normally infect cells by fusion with the plasma membrane of cells (Figure

3.1.1). Influenza virus only displays a capacity for fusion to lipid membranes under acidic conditions, optimally between pH5 and pH5.5 (Maeda and Ohnishi, 1980; Huang et al., 1981a). Virus-induced lysis of erythrocytes leading to the leakage of haemoglobin (haemolysis) has been used as a convenient experimental approach to measure fusibility of viral envelopes. The affirmed dogma is that a consequence of fusion of viruses with cell membranes can be leakage of molecules across the membrane (Poste and Pasternak, 1978). Although definitive evidence that virus-induced haemolysis is associated with virus-erythrocyte fusion has only so far been obtained for Sendai virus (Lyles, 1979) fusion is generally regarded as necessary for viral haemolysis. Consistent with this erythrocyte-erythrocyte fusion has been observed in association with viral haemolysis (Maeda and Ohnishi, 1980). The ability of influenza virus to cause haemolysis is dependent on acidic pH and a temperature above 10°C (Huang et al., 1980; Lenard and Miller, 1981). It would not be expected that influenza viruses would enter cells by fusion since the surface of cells is not acidic. However, influenza A/fowl plague virus/Rostock (H7NI) (FPV/R), which was prevented from undergoing endocytosis by being bound to a substratum can infect cells (through fusion with the plasma membrane) at neutral pH (Huang et al., 1981a).

It has been proposed that the fusion ability of influenza virus is brought into play when viruses reach the acidic environment of the endosome following its initial endocytosis

Figures 3.1.1 and 3.1.2. Postulated early events of the interaction between cells and influenza virus:

Figure 3.1.1. Schematic diagram of entry of influenza by direct fusion through plasma membrane.

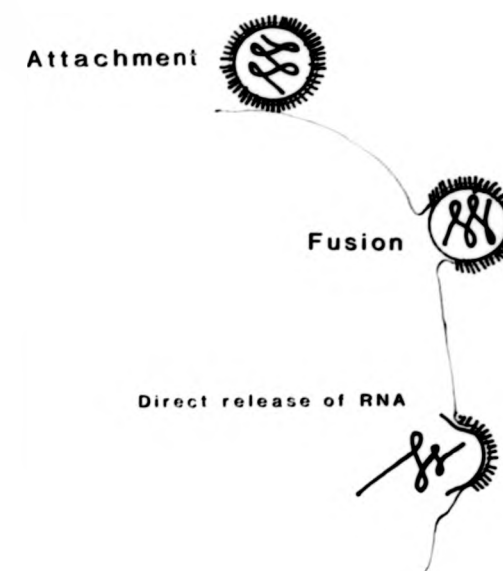
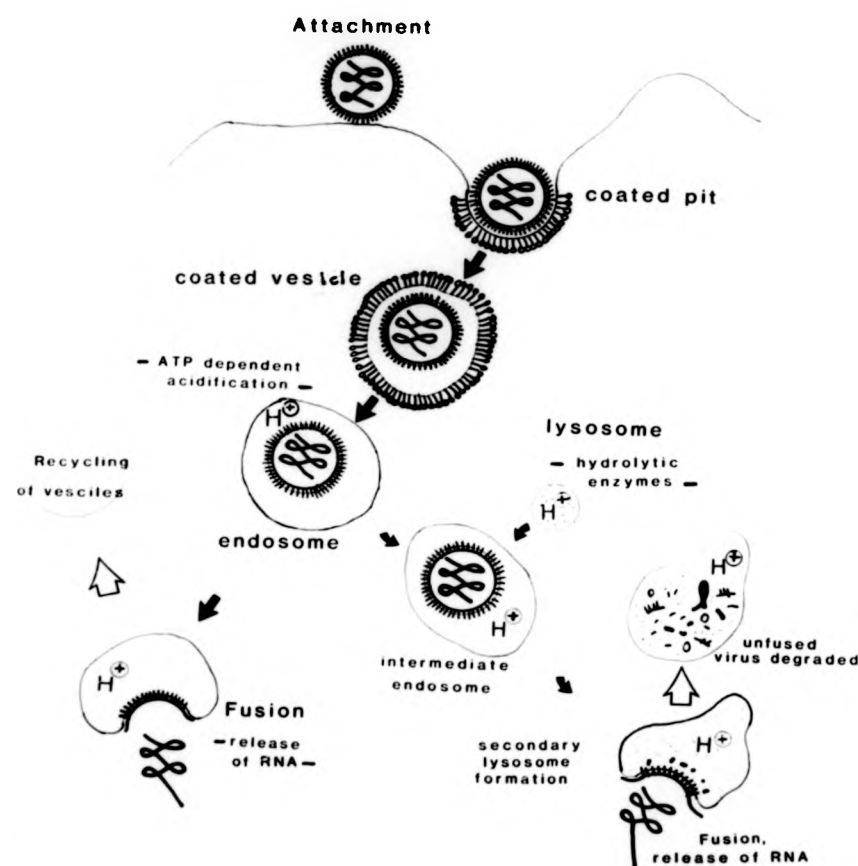


Figure 3.1.2. (Opposite) Schematic diagram of the adsorptive endocytosis pathway of entry leading to fusion in secondary lysosomes at low pH inducing the release of viral RNA.

Virus attaches to CRUs on the plasma membrane. Endocytosis is initiated at clathrin coated regions of the plasma membrane which pinch off to form coated vesicles\*. These vesicles lose their coats and deliver their contents to endosomes. Penetration takes place here dependent on acid-triggered conformational changes in the HA (section 3.3). Endosomes are a heterogeneous population of endocytic vacuoles through which internalized virus may pass on route to secondary lysosomes. The individual endosome matures and upon secondary lysosome formation acid-triggered conformational changes must lead to penetration before the hydrolytic enzymes degrade any unfused virus.

\* Influenza virus also observed to enter via smooth surface vesicles.

Diagram based on interpretation of experiments described by Matlin et al., (1981); Patterson et al., (1979); Maeda and Ohnishi (1982) and Yoshimura and Ohnishi (1984).

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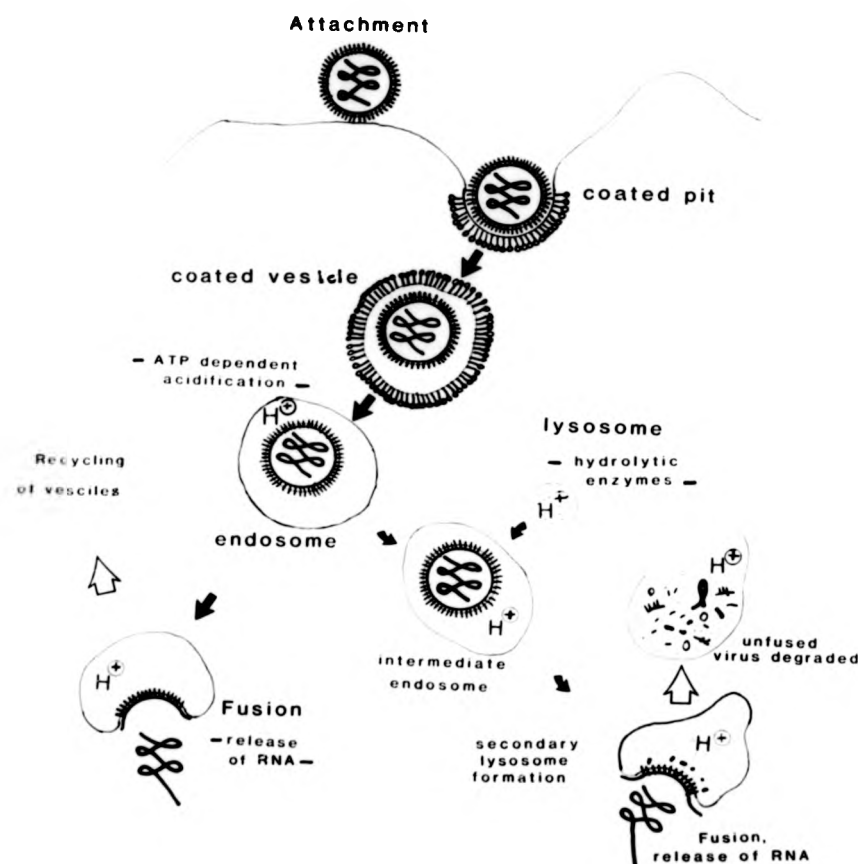
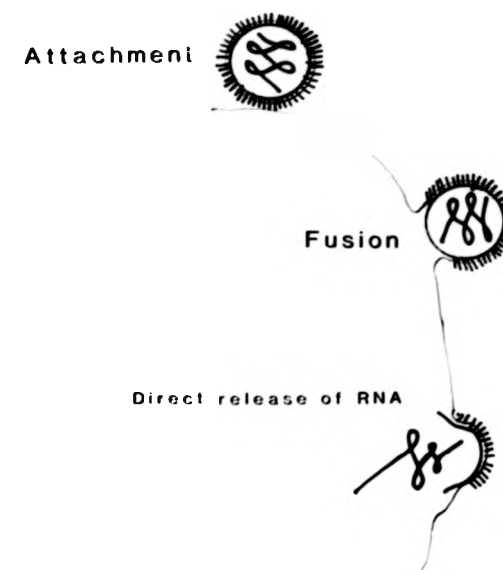


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(Figure 3.1.2) (Dourmashkin and Tyrrell, 1970; Patterson et al., 1979) and based on evidence of the low pH-induced fusion activity of influenza virus (White et al., 1981; Maeda et al., 1981; Yoshimura et al., 1982).

Virtually all mammalian cells are continuously engaged in phagocytosis, pinocytosis and adsorptive (receptor-mediated) endocytosis. Adsorptive endocytosis is a process for the uptake of essential macromolecules and if influenza virus enters by this mechanism it would be utilizing a normal cellular process to invade the cell.

Most of what is known about the pathways involved in the receptor-mediated uptake of enveloped viruses comes from the studies of Semliki Forest virus (SFV) by Helenius et al. (1980). SFV particles bind via viral glycoproteins to CRUs (particularly on microvilli) which are then concentrated in the locality. The virus then moves down into specialized regions of the cell membrane known as coated pits, the principle distinguishing features of which are the coating of clathrin molecules on their intracellular surfaces. Clathrin is a fibrous protein and takes the form of a polyhedral lattice or net for, and under, the receptors in the pit. The pits fold inwards to produce coated vesicles. The coated vesicles enter the cytoplasm, form into acidic prelysosomal vacuoles (endosomes) which can later form secondary lysosomes when they combine with lysosomes. Matlin et al. (1981) found that this sequence of events of internalization

by endocytosis, sequestration into intracellular vacuoles and secondary lysosome formation is also seen with influenza virus using MDBK cells (Figure 3.1.2). In addition to coated pits and vesicles, influenza virus particles were found in uncoated depressions in the plasma membrane and smooth uncoated vesicles were observed completely filled with virus particles. The importance of these smooth surfaced pits and vesicles to influenza virus entry is unclear. They may be caused by the plasma membrane progressively wrapping itself around the virus particles as more HA spikes are bound. Indeed, their formation may be induced by the virus particle itself. Patterson et al. (1979) noted that the majority of influenza virus particles were taken into cells in uncoated pits. SFV only entered BHK-21 cells in coated pits (Helenius et al. 1980).

EM observations by Patterson et al. (1979) showed that after attachment of influenza virus to chick embryo fibroblasts particles were endocytosed. After attachment the virus is gradually surrounded by the fluid cellular membrane. This process followed a pattern predicted by the thermodynamic analysis of the binding of polyvalent ligands to cell membranes (Hewitt, 1977) and influenza virus with repeating HA spikes may be regarded as a polyvalent ligand. Binding induces a curvature in the membrane which is concave towards the side to which ligand is bound. Invagination of membrane following virus attachment may be considered as the most thermodynamically stable configuration for the membrane to adopt. Thickening of the plasma membrane was

associated with the attachment site and this appeared to be internalized with virus giving rise to a vesicle with a thickened membrane. This method of entry was also observed by Matlin et al. (1981) illustrating that influenza virus does enter cells via smooth surface vesicles. Some vesicles contained more than one virus.

Adsorptive endocytosis unlike macropinocytosis and phagocytosis does not require energy (Allison and Davies, 1974). It has been found in electron microscopic observations that influenza virus endocytosis is unaffected by cytoskeletal inhibitors such as colchicine and cytochalasin B (Dourmashkin and Tyrrell, 1970; Patterson et al., 1979) illustrating that influenza virus entry does not require metabolic energy. Influenza virus also has the ability to enter lymphocytes and other cells at 4°C (Hackeman et al., 1974; Kato and Eggers, 1969; Stephenson and Dimmock, 1975; Stephenson et al., 1978; Mark et al., 1979, Possee and Dimmock, 1981). Micropinocytosis of colloidal gold occurs at 4°C in chick embryo fibroblasts (CEF) (Stephenson et al., 1978). Uptake at 4°C was similar to that at 37°C. The uptake of low density lipoprotein (LDL) into CEF cells by adsorptive micropinocytosis was also found to be temperature independent (N.J. Dimmock, personal communication). Adsorptive micropinocytosis (endocytosis) is the mechanism which accounts best for the penetration of infectious viruses at 4°C.

There are alternative patterns of processing (Hopkins, 1983)

of ligands apart from the route to a secondary lysosome and not all molecules that enter via coated pits reach lysosomes. For example, there are shuttle systems that return ligand and protein to the cell surface as in the case of transferrin (Daultry-Varsat et al., 1983; Klausner et al., 1983) or route the endocytosed molecule elsewhere, e.g. various hormones, apotransferrin, polymeric IgA and maternal IgG (Goldstein et al., 1979; Daultry-Varsat et al., 1983; Klausner et al., 1983; Rodewald, 1980). Experiments reported by Yoshimura and Ohnishi (1984) show that the majority of influenza virions could be uncoated in endosomes before reaching secondary lysosomes and prelysosomal fusion is accepted as the method of penetration of SFV (Marsh et al., 1983; Marsh, 1984). The heterogenicity in processing and endosome structure indicates that an invading influenza virus particle may be routed in various pathways.

The bias of current thought is therefore that the major site of entry of enveloped viruses is in the endosome (Marsh, 1984). Thus, virus would not be exposed to the destructive hydrolytic enzymes in the lysosomal compartment (Dales, 1973; Lonberg-Holm and Phillipson, 1974). It should be stressed that secondary lysosomes could serve a role in the processing of internalized influenza virus (Matlin et al., 1981).

### 3.3. Uncoating.

Evidence suggests that influenza virus enters the cytosol by fusion with the endosome or secondary lysosome membranes (Figure 3.1.2). The results of recent investigations have indicated that for a number of viruses, fusion occurs optimally over a narrow range of pH, which in the case of influenza viruses lies between 5 and 5.5 (White and Helenius, 1980; Vaananen and Kaariainen, 1980; Maeda and Ohnishi, 1980; Huang et al., 1981a) and it is proposed that this correlates with the pH at the site of cell entry in intracellular vesicles such as endosomes and secondary lysosomes (Review: Marsh, 1984). Acidification (due to the activity of a  $H^+$ -ATPase) of the endosome results in fusion of the viral membrane with the endosomal membrane and exclusion of the viral RNA into the cytoplasm. The penetration of SFV within endosomes enables the virus to accomplish fusion before entering the hydrolytic lysosomal compartment (Marsh et al., 1983). Other evidence put forward by Helenius et al. (1980) and White et al. (1981) for pH dependent fusion in the endosome or in the lysosome is that lysosomotropic agents (compounds that accumulate in endosomes and lysosomes) that also raise pH such as chloroquine,  $NH_4Cl$  and methylamine are efficient inhibitors of virus infection (Review: Dimmock, 1982). The multiplication of influenza virus is inhibited by lysosomotropic agents such as  $NH_4OH$ ,  $NH_4Cl$  and amantadine (Eaton and Scala, 1961; Fletcher et al., 1965; Hoffman et al., 1965). Amantadine interferes with a stage shortly after attachment and before any virus directed synthesis can occur (Skehel et al., 1977) and it is suggested that it inhibits fusion. Further, Bukrinskaya et al. (1982)

reported that rimantadine, an amantadine analogue, blocks influenza virus infection by preventing the release of M-protein during uncoating.

The ability of these lysosomotropic agents to raise endosomal or lysosomal pH, however, does not correlate with their degree of inhibition and compounds such as chloroquine might inhibit another process. The paramyxovirus, Sendai, fuses readily with cell membranes at neutral pH, but was inhibited at a similar early stage in infection by all lysosomotropic agents tested at similar concentrations to those required for inhibition of SFV and influenza virus (Miller and Lenard, 1981). This indicated that lysosomotropic agents could inhibit another stage of infection. The inhibitory activity of lysosomotropic agents on virus infection cannot be automatically interpreted as evidence that endosomes or lysosomes are the site of penetration (Dimmock, 1982). With SFV it was found that fusion of virus adsorbed to viral envelopes occurred after brief treatment of cells in the presence of chloroquine which resulted in an infection as effective as that that occurred in the absence of chloroquine, showing that the chloroquine does not effect the fusion reaction itself (Helenius et al., 1980).

Edwards and Brown (1986) found that Sindbis virus mediated fusion of cultured cells is a two step event. The virus must first be exposed to pH 5.3 (inducing a conformational change in the virus glycoprotein) but the fusion event itself requires a

shift of pH to neutrality. The requirement for a shift to a higher pH indicates either a second change in virus particles at that pH or some other requirement placed on fusion by the host cell. Chloroquine and  $\text{NH}_4\text{Cl}$  do not prevent the penetration of Sindbis (Cassel et al., 1984). These results suggest that passage through an acidic intracellular compartment may not be essential for penetration of cells by these viruses.

The M protein in influenza virus may play an important role in uncoating. It is tightly associated with both the envelope and the nucleocapsid (section 2.5). Its association with nucleocapsids causes inhibition of RNA synthesis in influenza virus (Zvonarjev and Ghendon, 1980). It seems likely that the viral nucleocapsid must free itself from M protein after fusion before it can start transcription. In view of the association of M protein with the viral envelope this decoupling process may well constitute a second step in uncoating. It has been suggested that amantadine acts in highly sensitive strains of influenza virus by potentiating the M protein-mediated inhibition of viral RNA polymerase (Zvonarjev and Ghendon, 1980; Lenard et al., 1982) and this may act on the second rather than first uncoating step at the low concentrations that effectively inhibit these strains.

Bukrinskaya et al. (1982) found that the uncoating of influenza virus proceeds in two major steps. Firstly, it is proposed that the outer lipid membrane of the virus is removed along with the embedded glycoproteins. This results in sub-viral

particles (SVP) that contain M protein and the RNP complex. In this respect, SVP are similar to the virus cores obtained by the treatment of influenza virus with detergents (Schulze, 1972). The results further implied that SVP are transported to the nuclear membrane where the second step in uncoating occurs; the removal from the M protein of the RNPs releasing naked RNPs. These steps take place in different cellular compartments. Intact virions were found in the cytoplasm, while SVP and RNPs were found in the nuclear associated cytoplasm. Rimantadine prevented the second step of uncoating, preventing the accumulation of SVP in the nuclear associated cytoplasm.

HA has now been directly implicated in membrane fusion as cloned HA can cause fusion without the presence of any other viral proteins (White et al., 1982). This confirmed previous conclusions from a variety of observations that HA is also involved in this activity. Post-translational cleavage of precursor HA (HA0) to HA1 and HA2 is required for both virus infectivity (Klenk et al., 1975; Lazarowitz and Choppin, 1975) and *in vitro* virus mediated fusion (Huang et al., 1981a; White et al., 1981). The amino terminal sequence of HA2 consists of 10 uncharged hydrophobic amino acids (Skehel and Waterfield, 1975) and it has been suggested that this region is involved in the membrane fusion reaction. The hydrophobic amino terminus of HA2 is analogous to that of the amino terminus of the F1 virus glycoprotein component of Sendai, a paramyxovirus that enters cells as a consequence of fusion of virus and cell membranes



(Scheid et al., 1978; Gething et al., 1978). This sequence is located 10nm from the distal tip of the HA molecule and 3.5nm from the viral membrane. The significance of this sequence in fusion was highlighted by the demonstration that synthetic peptides mimicking the appropriate sequence of the HA2 N-terminus inhibit virus induced membrane fusion and virus penetration, albeit very poorly (Richardson et al., 1980). Skehel et al. (1982) demonstrated that a conformational change in bromelain released HA (BrHA) occurs in the pH range 5.2-4.9, and after incubation at low pH BrHA is able to form hydrophobic associations with other BrHA molecules, lipid vesicles and nonionic detergent miscelles. It is envisaged that a molecular rearrangement occurs to allow direct action of the hydrophobic region of the HA2 (normally hidden in the stem region) with lipid membrane. The only charged amino acids in the first 18 residues of HA2 of influenza A/fowl plague/Rostock/34 (FPV/R) are two glutamic acid residues (Porter et al., 1979; Maeda and Ohnishi, 1980). If these residues are protonated (as might occur in the lysosome) the amino terminal segment would become uncharged and possibly hydrophobic enough to induce membrane fusion. As yet direct evidence of a causal relationship between the conformational change and fusion activity *in vivo* is lacking.

Huang et al. (1981b; 1985) maintain that both cleaved HA and NA activity are required for influenza virus cell fusion and haemolysis. Anti-NA antisera inhibited the fusion of erythrocytes and partially reduced haemolysis, but this was restored by the

addition of *Vibrio cholerae* neuraminidase (Huang et al., 1985). It was found that erythrocytes of various species showed different optima for haemolysis by FPV/R and further that erythrocytes could be sensitized for fusion and haemolysis by FPV/R at neutral pH if they had been pre-treated with low pH buffer. It was also found that not only virus proteins but cellular membranes undergo changes at acidic pH, since erythrocytes were fused by an acid wash. These data illustrated that the surface properties of erythrocytes are important in the low pH dependent fusion and haemolysis by influenza virus. Therefore, the acid pH activation of the virus is not obligatory for fusion. Acid treatment of the virus alone destroyed its ability to haemagglutinate and also caused irreversible viral aggregation. Haywood and Bayer (1985) demonstrated that influenza virus membranes are capable of fusion with liposomes at a range of pH. These data question the dogma that fusion in endosomes or secondary lysosomes is the only route of entry of enveloped viruses into cells (Helenius et al., 1980; White et al., 1981).

The observations discussed above can be unified if it is considered that influenza virus enters after interactions of HA and CRUs. Uptake is initiated and events ensue that destabilize the influenza virion sufficiently to release the influenza virus RNP into the cytosol which is rapidly transported to the nucleus and transcriptional replication of the vRNA occurs. The route by which influenza virus is transported to the nucleus is not known but travel is not likely to be random as 50% of the cell

associated input RNA is found associated with the nucleus after 15 minutes (Stephenson and Dimmock, 1975). A virus may travel directly through the network of membranes that comprise the cytoplasm or travel via the cisternae. Microtubules (Allison and Davies, 1974) might be responsible for directing vesicles containing influenza virus from the plasma membrane to the nucleus.

### 3.4. Transcription and replication of influenza virus.

Influenza virus replication is dependent on an active host cell nucleus both for viral transcription and for the processing of the viral mRNAs. The major host cell nuclear-dependent function involved in influenza virus replication is the synthesis of host cell mRNA which serves as a primer in the transcription of virus specific mRNA (Bouloy et al., 1978; Krug et al., 1979). The initial transcription of viral RNA in the cell to form mRNAs [poly A(+)cRNA] is known as primary transcription. This system can be readily duplicated *in vitro* using disrupted virions.

Secondary transcription is dependent on protein synthesis and results in progeny RNA molecules. Here the virus copies its vRNA into full length cRNA transcripts [poly A(-)cRNA] which act as templates for an RNA replicase to produce full-length vRNA for transcription into progeny virus. The synthesis of the three different classes of viral RNAs (mRNA, poly A(-)cRNA and vRNA), and the relative abundance of specific RNAs and vRNAs are controlled throughout infection.

#### 3.4.1. Early intracellular events: mRNA formation.

The first phase of influenza replication - primary transcription - results in the synthesis of all eight mRNAs

complementary to each segment of RNA in approximately equimolar amounts. In less than 20 minutes after infection mRNAs can be detected (Hay et al., 1977; Barrett et al., 1979; Mark et al., 1979) and their rate of synthesis reaches a peak after 2 hours. Primary transcription is independent of protein synthesis and takes place in the nucleus (Herz et al., 1981). Subsequently transcripts are transported to their site of function in the cytoplasm.

In order for transcription to take place cellular mRNAs are required. Virion RNA transcription *in vitro*, catalyzed by the virion-associated transcriptase, is greatly enhanced by the addition of a primer dinucleotide, ApG or GpG (McGeoch and Kitron, 1975; Plotch and Krug, 1977; 1978). *In vitro*, eukaryotic mRNAs and other RNAs containing a fully methylated cap structure (Gm<sup>7</sup> GpppX<sup>m</sup>) stimulated the transcription of influenza viral RNA (Plotch et al., 1981). 5 terminal RNA fragments containing the cap and the ensuing 10-14 nucleotides are cleaved from the host mRNA (e.g. beta-globulin mRNA) and are used as primers to initiate viral RNA transcription. The virion contains a cap-dependent endonuclease that cleaves capped RNAs preferentially at a purine residue that is 10-13 nucleotides from the 5 terminus. The mRNAs therefore have a 5' terminal type I cap structure (Krug et al., 1976). The priming of influenza viral RNA transcription by capped host RNAs also occurs in the infected cell (Krug et al., 1979). Host cell mRNAs and/or the precursors serve as primers for viral RNA transcription in the infected cell

and donate their cap and 10-15 nucleotides, one of which is m<sup>6</sup> A, to resulting viral mRNA molecules.

The 5' ends of influenza viral RNAs have to be donated from newly synthesized capped host RNA species, as pre-existing RNAs in the nucleus remaining after the addition of alpha amanitin do not prime (alpha amanitin inhibits DNA-dependent RNA polymerase II). Two of the influenza mRNA species are further modified by splicing (presumably by host cell enzymes) which allows overlapping regions of the genome to direct synthesis of additional polypeptides. In this manner NS<sub>2</sub> is derived from segment 8 and M<sub>2</sub> is derived from segment 7 (Lamb and Lai, 1980, Lamb et al., 1981).

The process of cleavage of the capped primer RNA, initiation, elongation and termination/polyadenylation of the transcript are all carried out by the proteins of the viral core (Plotch et al., 1981) i.e. NP, PB1, PB2 and PA (sections 1.3; 2.1; 2.3). NP is considered to have a structural role, PB2 is the cap recognition protein (Ulmanen et al., 1981; Braam-Markson et al., 1985). PB1 probably catalyzes the initiation of transcription (Ulmanen et al., 1981) and then acts as the transcriptase enzyme, adding nucleotides. It has not been determined which viral protein(s) act as the endonuclease (i.e. cleaving the capped RNA) but PB2 (in recognizing the 5' cap) and PB1 (in perhaps cleaving the phosphate bond) may constitute the endonuclease component. Although the PA protein is part of the

transcriptase complex catalyzing mRNA synthesis that moves down the growing viral mRNA chain (Braam et al, 1983), a specific function for the PA protein has not yet been identified.

#### 3.4.2. Template poly A(-)cRNA synthesis.

A second population of RNA transcripts [poly A(-)cRNA] are detected after the mRNAs appear in the cell and are synthesized whilst mRNAs are being produced. Synthesis of poly A(-)cRNA reaches a maximum rate 30-90 minutes post infection, before the peak of mRNA production. Poly A(-)cRNA molecules are complementary to the vRNA segments found in infected cells. They are complete transcripts of the vRNA segments, lack host derived primer sequences, are non-polyadenylated and probably act as templates for vRNA synthesis. The full length transcripts are probably also synthesized in the nucleus (Herz et al., 1981). As the synthesis of these transcripts is inhibited by cycloheximide it is suggested that synthesis is dependent upon a virus-specified protein(s) which perhaps modify the transcriptase complex so that transcription is initiated without a primer.

#### 3.4.3. vRNA Synthesis.

Naturally enough the synthesis of vRNAs follows poly A(-)cRNA production, although the precise timing of events is uncertain. The actual mechanisms by which vRNAs are replicated is not understood but synthesis is dependent on viral protein (Hay et al., 1977; Barrett et al., 1979; Mark et al., 1979). The

location of the site of synthesis of vRNA has also not been delineated. Segments can be detected both in the cytoplasm and the nucleus. vRNA segments are produced in non-equivalent amounts.

There is a correspondance between vRNA and mRNA synthesis suggests that the two events may be closely coupled (Smith and Hay, 1982). The time at which cRNA is synthesized suggests that the the template for synthesis of mRNA in secondary transcription is newly synthesized vRNA and that cRNA is synthesized only from input genome RNA (Wolstenholme et al., 1980).

The characteristics of influenza virus replication can differ depending on the host cell (Smith and Hay, 1982). There are also differences between virus strains (Lamb and Choppin, 1977; Hay et al., 1977). Sub-genomic RNAs which interfere with subsequent virus multiplication are sometimes synthesized during replication. These are defective-interfering (DI) RNAs (Nayak and Chambers, 1985). The generation of DI RNAs is favoured by high multiplicity and the host cell (Choppin and Pons, 1970; Janda et al., 1979) which may reflect a function provided by the host cell for replication.

### 3.5. Translation and processing of viral proteins.



Approximately 2 hours post infection NS<sub>1</sub>, NP and P proteins are detectable in infected cells and the M<sub>1</sub>, HA and NA proteins appear a little later (Skehel, 1972). Viral mRNAs bind to ribosomes in the cell cytoplasm and are translated into proteins in essentially the same fashion as eukaryotic mRNA, utilizing cellular tRNAs and initiation factors. The reliance of virus on normal cell processes continues in the modification and transportation of the spike proteins. The processing of HA glycoprotein (section 2.2), for example, exploits existing cellular pathways such as those normally used for the synthesis of secretory and membrane glycoproteins.

The biosynthetic pathway of NA is less well characterized, but glycosylated NA has been found associated with the endoplasmic reticulum and intracellular migration and further glycosylation has been observed. NA is inserted via an N-terminal hydrophobic domain anchoring the protein in the lipid. The anchor peptide also acts as a signal peptide during membrane translocation but is not removed by a signal peptidase (Fields et al., 1981).

### 3.6. Virus assembly and budding.

The newly synthesized virus coded polypeptides are transported to, and are assembled into, virions at the plasma membrane. The assembly of virus coded polypeptides at the plasma membrane is a specific and highly organized process as viral

products are found in discrete patches (Compans and Dimmock, 1969; Klenk et al., 1970) and no cellular proteins are detected in the mature virus (Holland and Kieln, 1970). Glycoproteins have to be inserted into the plasma membrane before virus assembly can begin (Lenard and Compans, 1974). The NP, P proteins and M proteins become attached to the cytoplasmic face of the plasma membrane only where there is a patch of spike proteins on the opposite side of the membrane. The mechanism of incorporation of the eight RNAs has not been determined there may be specific sites of interaction between the proteins and RNA which result in the enveloping of eight different RNA gene segments. The virions then bud (exocytosis) from the cell surface. It would appear that the enzymatic activity of neuraminidase ensures release of virions since antibody to NA aggregates virus at the cell surface (Dowdle et al., 1974; Webster et al., 1982). However, anti-HA antibodies are equally effective at preventing virus release (Dowdle et al., 1974)

#### 4. Influenza infection and pathogenesis.

Pathogenicity, i.e. the property of a virus to induce clinical disease, is the result of complex interactions between the infecting virus and the host organism. A number of factors usually intervene before clinical signs become manifest. Thus, virus-host interactions vary widely as do their pathological consequences. Some viruses always cause acute self-limiting disease, or subclinical infection. Others produce chronic infections, in which the virus persists for months or years before clinical manifestations. Epidemic influenza in man produces attack-rates which vary with age and type of community concerned. There is a great deal of variation in both the severity of illness in different persons and in the proportion of those with complications (Pereira, 1979). Influenza A viruses are not stable agents like many other viruses but undergo continual antigenic variation. This explains why persons of all age-groups can be involved in an epidemic because immunity which might be solid enough against a given influenza strain becomes inadequate if the haemagglutinin is altered. Attack-rates are therefore higher after a new subtype has appeared or a major degree of antigenic drift has occurred.

##### 4.1. Influenza, the disease and the inflammatory response to influenza infection.

To initiate infection of the human respiratory tract,

influenza has to combat non-immune defence mechanisms (Review: Sweet and Smith, 1980). Inhaled virus particles attach to epithelial cells via the HA spikes which bind to receptors on the cell containing N-acetylneuraminic acid. The mechanisms by which influenza initiates infection in cell culture are discussed in section 3, however a comprehensive description of the comparable events in the body has not been made. The ciliary beat, flow of secretions and the mechanical actions of breathing would help in preventing the initial contact between virus and respiratory tract cells. Once infection has been established in the absence of an immune response (section 5) these systems are counteracted to facilitate further infectious spread of virus. Uncomplicated influenza in previously healthy adults is confined to the epithelial and sub epithelial layers of the mucosa of the respiratory tract (Walsh et al., 1961) with occasional secondary involvement of the lungs and rarely viraemia (Sweet and Smith, 1980). Influenza can destroy the ciliary activity of the trachea and the necrosis of superficial epithelial cells restricts the self-cleansing of lungs, as the upward movement of the mucous blanket is inhibited (Walsh et al., 1961; Ebisawa et al., 1969). This decreases the clearance of virus. Secretions over the mucosa increase as an inflammatory response to destruction of cells and the beat of those cilia that remain is less effective (Ebisawa et al., 1969; Sweet and Smith, 1980). Inflammation is typified by the migration of polymorphonuclear leucocytes (PMN), monocytes and mast cells to the site of infection along a chemotactic gradient. Serum components such as the kinin system and

vasoactive amines produced by mast cells contribute to inflammation (Burrows, 1972; Mims, 1972; Sweet et al., 1977). Lysosomal enzymes from the lysosomes of PMNs cause erythema and irritation. Lung and peritoneal macrophages obtained from unprimed mice are able to inactivate influenza viruses (Rogers and Mims, 1981) but the contribution of macrophages in this manner may be minimal because of lack of direct access to infection sites. PMNs have a half-life of 6-7 hours (Stossel, 1974) and their degeneration causes the gross secretion in the respiratory tract to become purulent (Elsbach, 1973). In response to the arrival of virus and viral products, the lymphoid tissues induce further inflammation and early immune responses (section 5). Influenza in man can also affect the chemotactic and phagotactic activity of macrophages and PMNs (Kleinerman et al., 1975; Larson and Blades, 1976). This, compounded with the necrosis and exposure of underlying tissue encourages bacterial invasion and disease.

The dry coughing and sneezing of the earlier phases of illness will also carry the infection to other areas (Review: Stuart-Harris 1965). This stage is followed by a stuffy, blocked nose and later by the expectoration of mucoid or mucopurulent sputum from degenerating PMNs. The fever that occurs in influenza infection in man is accompanied by headache, weakness myalgia in limbs and back and can last from 24 hours to 5 days in more severe infections. Body temperature during fever may range from 38-40°C. (Stuart-Harris et al., 1985; Fenner et al., 1974).

Nasal secretions contain non-specific inhibitors of the HA and infectious activity of influenza which by nature are mucoid (Review: Shvartsman Ya and Zykov, 1976). The activity of these inhibitors is destroyed by the addition of *Vibrio cholerae* or influenza neuraminidase. The thermolability can differentiate between virus infectivity inhibitors which lose activity at 56°C after 60 minutes and anti-haemagglutinin inhibitors which are stable at 70 °C. These inhibitors neutralize a wide range of influenza virus A strains, influenza B and Newcastle disease (Shvartsman Ya and Zykov, 1976) and are probably critical factors of non specific immunity to influenza.

The experimentally induced influenza virus infections in ferrets is closely analogous to the pathological effects in man (Sweet et al., 1979). Fever in the ferret is associated with the release of a pyrogen from leucocytes. Therefore before overt effects in the lung influenza virus must overflow systemically. But it has been difficult to demonstrate viraemia. In humans it is thought that the virulence of influenza is relatively fixed with the severity of the disease being linked to the condition of the human host (Kilbourne, 1960), however experimental influenza in ferrets varies greatly during adaptation from human specimens (Toms et al., 1977; Matsuyama et al., 1980).

Influenza in man can be complicated by pneumonia caused by *Staphylococcus pneumoniae* and *S. pyrogenes* (Giles and

Shuttleworth, 1957; Robertson et al., 1958). There is aetiological evidence for links between encephalitis lethargica and post-encephalitis Parkinson disease (Flewett and Holt, 1958; Ravenholt and Foege, 1982). Associations have been observed between cardiac complications (Hers et al., 1958; Louria et al., 1959).

The epidemiology of influenza in the past has provided important insights into the disease and epidemiological surveillance remains a crucial factor in the control and understanding of human influenza (Reviews: Stuart-Harris, 1979; Pereira, 1979). The majority of influenza infections have low morbidity in working adults and convalescence in these cases is usually rapid once fever has disappeared. Influenza can have high rates of infection amongst children under 5 (Hall et al., 1973) and in the elderly where high mortality rates may occur, particularly in geriatric residential homes (Stuart-Harris, 1979). High rates of infection can also occur amongst people in any large residential institution such as in schools and in army camps. This is perhaps due to increased transmission rates (Stuart-Harris, 1979) or an environmental factor that effects the group (Shope, 1944). Reemergence or recycling of a strain can have an influence on age specificity. For example, during the 1957 outbreak of A/Asian virus/57 (H2N2) the highest rate of attack occurred in children. The lowest rate was in adults over 70 years of age. This was because these adults had been exposed in childhood to infection by an influenza virus bearing the H2

haemagglutinin (Mulder and Masurel, 1958).

Influenza A viruses have also been shown to produce epidemics of respiratory disease in swine, horses, and seals. There are clinical similarities between swine, horse and human influenza. In avian species different strains of influenza produce strikingly different patterns of disease; ranging from a completely asymptomatic infection to a fatal epidemic disease as in the case of fowl plague where there is viraemia, CNS involvement and general dissemination (Stubbs, 1965). Influenza infection can be induced in laboratory mice by the intranasal inoculation of influenza. This initiates a pneumonic infection in the lungs and is characterized by a gross pathological effect. Influenza does not infect mice in the wild, and inbred strains of mice differ in their susceptibility to infection. For example, mice bearing the gene Mx survive challenge with strains of influenza that are usually lethal for non-Mx bearing mice (Haller et al., 1979). This resistance *in vivo* correlates well with an interferon-dependent resistance of infected cells. Although studies of influenza infection and immunity in mice (section 5) have provided information not available from humans the difference in pathology should always be considered.



#### 4.2. Molecular determinants of virulence and pathogenicity.

There is a correlation between the structure of the HA and pathogenicity of influenza (Rott, 1980). The ability of influenza to replicate in tissue culture or in the host is dependent on host enzyme cleavage of the HA (Bosch et al., 1981) and efficient cleavage of precursor HA polypeptide into HA1 and HA2 in a wide range of cells appears to be essential for the virulence of virus (Klenk et al., 1975; Lazarowitz and Choppin, 1975).

The amino acid sequence of the connecting peptide between HA1 and HA2 may determine the range of cells in which cleavage can occur. Highly pathogenic avian influenza viruses that cause generalized infections and sudden death in birds exist among viruses of H7 and H5 subtypes, while other avian viruses of other subtypes are relatively non-pathogenic despite being infectious. FPV/R (H7N1) causes a lethal infection in chickens and has the sequence Lys-Lys-Arg-Gln-Lys-Arg between the C terminus of HA1 and the N terminus of HA2 (Porter et al., 1979; Bosch et al., 1981) while non pathogenic avian influenza viruses of the same subtype (H7) contain only a arginine-glycine residue. Investigation of groups of pathogenic viruses, human viruses (which are avirulent in birds) and non pathogenic avian influenza viruses of the H7 subtype suggested that the HA molecules from human and non pathogenic avian viruses had fewer insertions in the connecting HA peptides than those of the pathogenic strains.

The long connecting peptide being susceptible to proteolytic removal in cells of a wide variety of tissues.

However the structure of the HA is almost certainly not the only factor determining pathogenicity. From studies on influenza reassortants obtained *in vivo* it was found that a large number of reassortants obtained from a highly pathogenic influenza for chickens and an non pathogenic virus showed that exchange of RNA segments modified pathogenicity. Increase or loss of pathogenicity seemed to depend not only on the HA but also on the influenza genes involved in viral synthesis, i.e. the nucleoprotein and PA genes. Recombinants obtained from non pathogenic viruses can assume pathogenic properties (Rott et al., 1979; Vallbracht et al., 1978; Scholtissek et al., 1979) or in contrast reassortment of genes between highly pathogenic strains can lead to non pathogenic reassortants as well as pathogenic viruses (Rott et al., 1976). These data indicate that an optimimal constellation of RNA segments is required for the genome of a highly pathogenic virus strain. Oxford et al. (1978), found that virulence of reassortants of a H3N2 virus in human volunteers covaried with genes for both HA and NA.

#### 4.3. Interspecies transmission.

Recombination events demonstrated *in vitro* may represent evidence for the derivation of new strains of human virus from recombination events in nature between animal or avian viruses.

This was proposed by Laver and Webster (1973;1979). For example, human epidemics frequently originate in S.E. Asia where densely populated rural communities live in close proximity to ducks and pigs. This ecosystem allows the possibility of genetic recombination to occur between influenza viruses of different species. In support of this concept, the Hong Kong (H3N2) virus contains the NA ( and other) genes that are almost identical to those from an asian (H2N2) strain of human influenza and a HA which is antigenically related to that of A/Duck/Ukraine/63 (H3N8) and A/equine/2/Miami/63 (H3N8) viruses. The amino acid sequence homology between the HAs of A/Duck/Ukraine/63 and A/Aichi/2/68 viruses (both H3) is 96%. The HA could have been donated during a recombination event in an animal or avian species and then transmitted to a human initiating the epidemic. Interspecies transmission has been implicated in the infection of humans of H1N1 influenza viruses derived from pigs at Fort Dix, New Jersey (Topp and Russell, 1977) and in Wisconsin (Hinshaw et al., 1978).

The A/Seal/Massachusetts/1/80 (H7N7) virus recovered from the lungs and brains of harbour seals off the north-eastern coast of the U.S.A. possessed close genetic homology to A/FPV/Dutch/27 (H7N7), a highly lethal influenza of domestic fowl not previously found in mammals (Webster et al., 1981a). Direct tropism for the brain has not been detected before in influenza in mammals. These events may represent evidence for the natural transmission of an avian influenza to a mammalian species although this remains to

be confirmed. The seal influenza virus has been associated with some cases of human conjunctivitis (Webster et al., 1981b) and an influenza that had originally been found in striped whales in the Pacific (H1N3) has been implicated in illness in humans (Lvov et al., 1983). These observations should deal with any complacency regarding influenza and that new influenza virus strains in humans could manifest a completely different pathology than that seen in the present circulating subtypes.

## 5. The immune response to influenza infection.

### 5.1. The molecular basis of influenza antigenicity.

The remarkable antigenic variability of influenza A viruses ensure the epidemic potential of influenza allowing new strains to circumvent the serum antibody to existing and previous virus strains. Variation within a subtype may be brought about by several mechanisms: Point mutations in genes coding for HA or NA and other proteins and reassortment of genes coding for other proteins. Variation leading to different subtypes may be due to reassortment of HA or NA genes or the reemergence of previously circulating strains. Monoclonal antibodies, because of their high level of discrimination have been used to show minor (insignificant) antigenic changes, resulting from point mutations, in matrix (M<sub>1</sub>) proteins (Van Wyke et al., 1980; Le Comte and Oxford, 1981), and in NS1 protein (Shaw et al., 1982). Schild et al. (1979) using polyclonal sera demonstrated minor antigenic differences between NP proteins. However, overall, it is clear that the NP proteins are serologically closely related and the relatively small antigenic differences may have no immunological or epidemiological significance.

It is the considerable antigenic variability of HA and NA that is the most relevant. The most significant proportion of protection against reinfection with influenza is due to neutralizing antibody against the HA (Hobson et al., 1972; Virelizier, 1975; Ennis, 1982). Virelizier (1975) demonstrated that specific antibody to HA transferred to naive mice in

physiological concentrations can passively protect both normal and immunodepressed recipient mice. The protective effect of anti-NA antibody is less than that of anti-HA antibody (Schulman, 1970; Potter and Oxford, 1979) although passively administered anti-NA does protect mice (Schulman et al., 1968). Antibody to HA acts by neutralization of virus particles whereas antibody to NA inhibits virus release from infected cells (Dowdle et al., 1974).

Animal experiments have established that antibody to M<sub>1</sub> or NP have no protective effect against challenge with influenza A viruses (Oxford and Schild, 1976).

## 5.2. Variation in the haemagglutinin.

The variation in the HA molecule of influenza occurs in two ways. Small changes in antigenicity (antigenic drift) are the result of a gradual accumulation of point mutations or short deletions/insertions in the HA gene, while complete change in antigenic properties (antigenic shift) involves the replacement of the gene coding for one HA with that of another.

### 5.2.1. Antigenic drift.

During the prevalence of an influenza virus A subtype minor changes take place in the HA (Review: Webster et al., 1982). The mechanism of antigenic drift involves the selection by immunological pressure of mutant viruses with altered antigenic

determinants (Laver and Webster, 1968). The structural changes leading from the accumulation of amino acid sequence changes allows new virus strains to be infective by evading neutralization by antibody present in the population. Sequence analysis of the HAs from naturally occurring variants of the H3N2 subtype from 1968-1979 show that most of the amino acid sequence changes have occurred in the HA1 region of the molecule (Webster et al., 1982).

The selection of escape mutants by culture of virus with monoclonal antibodies in eggs have also shown that substitutions occur in the HA (Gerhard and Webster, 1978; Laver et al, 1979; Wilson et al., 1981). Further, these studies have shown when the three dimensional structure is examined (Wiley et al., 1981) that the changes are clustered on the H3 HA into four non-overlapping antigenic regions A-D (Wilson et al., 1981 and section 2.2). The sequencing of the HA showed that only a single amino acid substitution was sufficient to prevent the monoclonal antibody in question from binding to the mutant (Laver et al., 1979). Other monoclonal antibodies recognizing topologically distinct antigenic sites could still bind and neutralize the mutants as could polyclonal antibody against the strain.

#### 5.2.2. Antigenic shift.

The term shift is applied to the emergence of a new influenza A virus possessing glycoproteins so different from

those of the prevalent viruses that they are designated as new subtypes. The peptide sequences of HAs of different subtypes show major differences that could not have arisen from mutation of the HA gene of the preceding virus. There is circumstantial evidence that the recombination events observed *in vitro* may occur in nature (section 4.3) and viruses from non-human hosts have been found to contain HAs related to H1, H2 and H3 human subtypes (Laver and Webster, 1973; 1979). Recycling of the antigenic subtypes may also occur. This could explain the recurrence of the H1N1 virus in 1977, twenty seven years after the last recorded appearance of a genetically identical H1N1 virus (Nakajima et al., 1978; Scholtissek et al., 1979). It is difficult to explain where this virus was or why it remained unchanged for so long.

There is an additional selective mechanism on the HA for generation of variants of influenza A viruses dependent on the host cell used for growth (Schild et al., 1984). The cultivation of influenza virus in eggs selects sub-populations of virus with HA antigenically distinct from that of virus grown from the same (human) source but in MDCK cells. Host cell selection pressure provides an additional (non-immune) mechanism for antigenic selection and could have epidemiological significance.

### 5.3. Variation in the neuraminidase.

Neuraminidase also undergoes antigenic shift and drift. In 1957 human influenza A viruses bearing N1 neuraminidase were



replaced by N2 in a shift event likely caused by reassortment and antigenic drift has been documented in the N2 NA since 1957 (Martinez et al., 1983). Animal viruses containing the N1 and N2 subtypes have been identified (Webster and Pereira, 1968; Schild and Newman, 1969). An operational map from the selection of variants with monoclonal antibodies has been constructed which defines at least four non-overlapping antigenic sites (Jackson and Webster, 1982).

#### 5.4. Immune response in recovery from influenza virus infection

A variety of interacting immune mechanisms may contribute to the recovery from influenza infection. Antibody, cell mediated immunity (T cells, macrophages, natural killer cells) and interferon all co-operate together to bring about recovery. The dependence on co-operative interplay between different cell types (often via soluble mediators) means that the assignment of a determinative role of a given cell may be impossible. Nevertheless, *in vivo* and *in vitro* experiments have provided a detailed analyses of the repertoire and potential of immune mechanisms.

McMichael et al. (1977) first demonstrated HLA restriction of cell mediated lysis of influenza virus infected human cells by T cells. Cytotoxic T cells (Tc) mediate this lysis via a lytic signal to infected cells and the phenomenon is demonstrated in cultured cells in a  $^{51}\text{Chromium}$  release assay for cytolysis. Target cells and Tc must be histocompatible. Tc recognize virally coded antigenic determinants on the surface of infected cells, in association with major histocompatibility antigens (Doherty and Zinkernagel, 1975). In mice the histocompatibility antigen is coded by the K, D or L gene of the mouse H2 gene and by the HLA-A, B or C in man (Review: Zinkernagel and Doherty, 1979).

There is now evidence that illustrates the important role of Tc in the recovery from influenza infection (McMichael et al., 1983a) where Tc activity correlated with reduced viral shedding in volunteers who lacked antibody to the HA or NA. Ennis et al.

(1981a; 1982) described the dynamics of Tc response in volunteers infected with influenza or vaccinated with inactivated whole virus or subunit vaccine. T cell cytotoxicity was very low 7 days post inoculation but was fully expressed between 14 and 21 days. By this time most volunteers had also produced good neutralizing antibody responses. Six months post infection or vaccination memory Tc activity had returned to preimmune levels. Yap et al. (1978) showed that the transfer of murine immune Tc to synergic recipients leads to a reduction of viral titres in their lungs. This observation has been reproduced with a cloned murine Tc line (Lin and Askonas, 1981).

Studies in mice have shown that T cell cytotoxicity develops early after the onset of influenza virus infection and reaches a peak level of cytotoxicity before antibody is detected (Doherty et al. 1977). Levels then return to baseline and are not detected in the resting state.

Newly synthesized virus coded proteins are present in the plasma membrane of infected cells, therefore Tc lysis of virus infected cells can occur before any progeny are released (Zinkernagel and Althage, 1977). The clearance of infectious foci from tissue sites of pathology requires the collaboration of monocytes or activated macrophages (Blanden, 1974). In the mouse model depletion of macrophages delays viral clearance.

It was found that the majority of Tc appear to be unable to

distinguish between influenza A virus subtypes (Zeewink et al., 1977, McMichael and Askonas, 1978) and investigation of cloned Tc lines have confirmed that the majority are fully cross reactive (Braciale et al., 1981; Fleischer, 1982). No cross-reaction has been demonstrated between A and B influenza types. Using target cells infected with variant and recombinant influenza viruses it has been shown that Tc may be strain specific for HA and that some Tc clones map to polymerase and NP genes (Bennick et al., 1982). NP is present on the membrane of infected cells along with the glycoproteins and the NP antigen, because it is antigenically common to all influenza A viruses (section 2.3), would represent an ideal protein for cross-reactive Tc to recognize. L cells transfected with cloned DNA for HA were recognized by a minor subpopulation of Tc and not by the cross reactive Tc (Townsend and Skehel, 1982). This illustrated that HA is not the antigen recognized by the majority of cross-reactive Tc. NP antigen transfected into L cells made these cells targets for Tc proving that NP alone can act as the target antigen (Townsend et al., 1985). Further functional diversity of Tc was indicated by Taylor and Askonas (1983) who found that some Tc with strong cytotoxic activity *in vivo* did not provide protection *in vitro*. Cross-reactive Tc would provide heterotypic immunity in contrast to neutralizing antibody which is strain specific for the HA.

Mitchell et al. (1985) report that HA specific Tc were not blocked by a panel of monoclonal antibodies directed to the HA showing that Tc and antibody recognize different epitopes. This

is probably a function of *in vivo* competition between high affinity antibody and T cell clones leading to the selection of Tc that recognize alternative epitopes.

The key event in virus elimination by Tc may not be cytotoxicity itself but the secretion of gamma interferon. It was found (Morris et al, 1982) that the ability to decrease the virus titres in the lungs of mice by adoptive transfer of cloned Tc correlated with the production of gamma interferon. On transfer into infected mice a Tc clone inhibited the replication of homologous or heterologous influenza virus. The same Tc clone released gamma interferon only when in contact with its matched target cell. This may be the event in virus elimination not cytotoxicity. Those Tc clones that produced little or no gamma interferon after appropriate antigenic stimulation did not clear virus from the lungs. It should also be considered that the functions of cytotoxicity and interferon release operate together

during influenza virus infection. Tc could therefore aid host defence by directly killing cells that express viral antigen on their surfaces and by producing gamma interferon. Gamma interferon has a direct effect as an inhibitor of viral replication and also increases target cell expression of MHC antigen making these cells more sensitive to Tc (Review: Rager-Zisman and Bloom, 1985). It also activates natural killer (NK) cells which mediate non-specific lysis of infected cells. Alpha interferon is also produced early in infection with influenza (Ennis and Meager 1981) and could contribute by making

epithelial cells resistant to virus infection and, with gamma interferon, increasing the removal activity of NK cells.

The generation of a potent Tc population requires the collaboration of the helper T cell (Th) subset (Zinkernagel and Doherty, 1979). Th recognize viral antigen presented in conjunction with the I-A (or I-E) gene product on macrophages. The regions that Th cells recognize on the HA have been mapped (Figure 2.1.2). Helper T cells play a crucial part in the generation of both antibody and Tc immune responses and are essential for recovery from viral infection (Mims and White, 1984). IL-1 secreted by monocytes/macrophages activates Th to secrete several lymphokines known as ThF. Antigen specific ThF trigger appropriate clones of B cells to differentiate into antibody secreting cells. There are subtype specific and cross reactive influenza Th clones (Lamb and Green, 1983).

T cells can have another effector function - induction of delayed type hypersensitivity (DTH) by a subset known as Td cells. DTH is a T cell mediated inflammatory reaction characterized by infiltration and proliferation of mononuclear cells and influenza DTH has been demonstrated in mice (Leung et al., 1980) and man (Habershon et al., 1973). The DTH reaction is eventually shut off by suppressor T cells. Inactivated influenza gives a greater DTH response than does live virus (Ada et al., 1981) illustrating that DTH is probably of importance in attracting macrophages and T cells in the early phase of the

immune response to viruses but needs to be suppressed after this task is completed. Suppressor T cells specific for influenza virus subtypes have been described and may be involved in regulation of influenza immunity (Liew and Russell, 1980).

NK (natural killer) cells are leucocytes that have the capacity to kill virus infected cells and to a lesser extent some normal cells by spontaneous cytotoxicity. Rager-Zisman and Bloom (1985) characterize the recognition of NK cells as selective for certain (as yet unknown) target structures. NK cells are partially regulated by interferon and all three classes of interferon can augment NK activity (Rager-Zisman and Bloom, 1985). Ennis et al. (1981b) showed that volunteers infected with influenza A develop increased NK activity within 1-2 days of infection and this was still elevated on day 6. The level of cytotoxicity of the NK cells correlated with the production of gamma interferon. Therefore there is suggestive evidence that NK cells are important in the defence of influenza virus infection before Tc and antibody emerge, but their role is still uncertain. Nude mice have high levels of NK activity which is further elevated by influenza but virus is not cleared unless there is adoptive transfer of H2 restricted T cells (Ennis, 1982).

Influenza virus infection is generally restricted to epithelial tissues (Stuart-Harris et al., 1985) and incubation is usually no more than a few days. The dynamics and evolution of an effective antibody response in man appears to lag behind the

course of an acute influenza infection, and indeed the infection may have already finished (Rossen et al., 1970). Three days after the infection of mice with influenza there is a great increase in the number of IgA and IgM producing plasma cells in the subepithelial tissues (Owens et al., 1981). This is followed a few days later by an increase in IgG plasma cells. During primary infection antibodies are not detected in secretions or in the serum until 8 days by which time the replication of influenza has been brought under control. These observations do not preclude the possibility that antibody is synthesized several days earlier but is in too low a concentration to be detected, or in this early phase of infection antibody could be complexed to viral HA and is only detected when in excess of antigen.

The most relevant site for antibody to participate in recovery from influenza, considering the route and site of infection, is perhaps at the mucosal surface of the upper respiratory tract (Review: Rossen et al., 1971). Neutralizing secretory IgA (sIgA) in the nasal washings of humans with influenza infections rose in titre up to a 10 day peak concomitant with a rise in serum IgG titre (Alford et al., 1967; Rossen et al., 1970). The sIgA titre returned to baseline levels after 25 days whilst the serum IgG titre remained at a high titre even after 28 days (Alford et al., 1967). Virus was not isolated in the nasal washings after the fourth day. These findings were essentially confirmed by Murphy et al. (1973) who observed a similar pattern of neutralizing sIgA production after first



detection 4-7 days after infection. Although sIgA is the predominant immunoglobulin in mucosal secretions significant amounts of other isotypes are found (Review: Dhar and Ogra, 1985) and evidence suggests that protection against mucosal infection in patients with IgA immunodeficiency may be effectively provided by the compensatory local production of virus specific IgM and IgG in the respiratory tract (Ogra et al., 1974).

Specific anti-influenza activity is observed in all immunoglobulins in the nasal secretions (Murphy et al., 1973). This is perhaps best illustrated by the observations of Murphy et al. (1982) who using a live attenuated vaccine strain studied the dynamics of antibody response in humans (as detected by enzyme-linked immunosorbent assay). In nasal secretions IgM and sIgA specific for influenza HA appeared simultaneously after 2 weeks, IgG detection occurred a week later. IgM peaked at 2 weeks, IgG at 4-6 weeks and the sIgA titre continued to climb into the 9th week. A sIgA response was seen in all individuals in the study, but the frequency of IgG and IgM was reduced. By two weeks post-infection IgA, IgG and IgM were all present in the serum. The activity of IgM and IgA in the serum was transient, peaking at two weeks then declining. IgG antibody in the serum increased in titre to reach a peak after 4-7 weeks.

The parallel rise of antibody in the serum and in the nasal washings of man (Murphy et al., 1982) and in the lungs of mice (Liew et al., 1984) suggest that serum antibody has a

contribution as significant as that of secretory antibody in the recovery from influenza virus infection. The dynamics of production of specific antibody indicate that antibody may aid in recovery only if the infection lingers on. However the barely detectable levels of antibody seen early in infection may be significant in immune mechanisms involving complement and antibody dependent cell-mediated cytotoxicity (ADCC).

The classical complement pathway (Review: Cooper, 1979) can be activated after the combination of antibody and antigen, and the first antibodies produced in infection could depend on complement for neutralization. This could result in damage or lysis to the envelope of influenza, deposition of complement proteins leading to prevention of attachment or activation of complement following the attachment of antibody to viral antigens on infected cells may result in the lysis of that cell. In addition it should be noted that complement can carry out inactivation of viruses without the participation of antibody via the alternative pathway (Review: Sissons and Oldstone, 1980). This is an important phenomenon because it can occur before immune responses have been generated. The alternative complement pathway can be directly activated by the classical complement pathway in an activation loop. Initiation of the classical complement pathway occurs when the Fc regions of bound IgM and some classes of IgG (in humans IgG<sub>1</sub> and IgG<sub>3</sub>) are recognized by C1q. There must be at least two Fc sites (i.e. there must be more than two IgG molecules close together). With IgM several Fc sites

are present on a single molecule, and therefore IgM can activate complement much more efficiently. Secretory IgA does not activate complement (Tomasi, 1976).

Antibody dependent cell-mediated cytotoxicity (ADCC) is a very efficient process that takes place in the presence of antibody by certain types of immune cell. These effector cells are not immune specific, they bind to target cells to which immunoglobulin is attached and such cells are destroyed. This can be mediated by cells such as PMNs, K lymphocytes (non-T, non-B) and macrophages bearing Fc receptors (FcR) for the Fc regions on immunoglobulins. As well as IgG, sIgA is a potent mediator of ADCC (Tagliabue et al., 1983). Greenberg et al. (1977) demonstrated that ADCC has a role in influenza virus infection. Haemagglutinin specificity was maintained and potent ADCC occurred when antibody concentration was too low to be assayed by neutralization assay. Indeed, ADCC was found by Shore et al. (1976) to be hundreds of times more efficient per antibody molecule than complement-mediated cytolysis. Therefore ADCC may be an important factor *in vivo* particularly in early infection phase.

In summary the immune response leading to recovery from infection with a new influenza strain is a complicated process involving a variety of humoral and cell-mediated mechanisms. There is an apparent need for immunologically specific T cells for recovery from influenza, but early control may be effected by

interferon and NK lymphocytes. Conclusions regarding the precise importance of each mechanism cannot really be made in isolation as all normally co-operate together, meaning that to some extent any attempt to assess relative importance is an artificial exercise. The early response may be crucial in aiding the host to limit and control virus infection until immunologically specific Tc are generated to destroy the virus infected cells. T cells which aid in the production of antibody are detected later in infection and neutralization by antibody appears to be more important in resistance (protection) to later challenge.

#### 5.5. Immune response in resistance to influenza virus infection.

The continual antigenic turnover of influenza A viruses (sections 5.1. to 5.2.2) accords short-lived resistance to further re-infection. The first (and perhaps major) line of defence of acquired immunity to re-infection with the same strain of influenza would appear to be antibody generated from anamnestic response from specific B lymphocytes (Reviews: Potter and Oxford, 1979; Couch and Kasel, 1983). However, it is apparent that cell-mediated reactions do occur in man and animal models and contribute to resistance (McMichael et al., 1982). Mechanisms by which antibody can mediate host resistance to virus infection are neutralization, opsonization for phagocytes, antibody-complement mediated effects and ADCC. The utilization of these mechanisms on the re-challenge of virus of the same strain would mean the end of the infection. The premise that neutralizing antibody offers prophylactic immunity assumes that if its surveillance is breached the mechanisms that contribute to recovery (section 5.4.) are brought into play.

Many studies have shown that a significant proportion of the protection mediated by the immune system against re-infection is due to the presence of neutralizing antibody against the HA (Laver and Kilbourne, 1966; Hobson et al., 1972; Virelizier, 1975; Reviews: Potter and Oxford, 1979; Ennis et al., 1982).

Antibody to NA has a less clear cut protective response in man (Potter and Oxford, 1979) and it seems most likely that anti-NA reduces the quantity of newly synthesized virus released from infected cells. This limits the spread of infection. There is no apparent correlation between neuraminidase inhibition by anti-NA antibody and resistance to infection (Hobson et al., 1972).

Antibody to HA need only be considered in respect to the other viral proteins as it satisfies the four "mediator constraints" of Couch and Kasel (1983). The overall polyclonal antibody response to influenza to be protective must possess: strain specificity; cross-reactivity within a subtype but reduced cross-reactivity for variants that are distinct from the initial strain; long term durability of immunity and activity at the respiratory surface. Serum haemagglutination inhibition (HI) antibodies can neutralize *in vitro* (Hobson et al., 1972) and inhibit virus release from infected cells (Dowdle et al., 1974). However direct correlation of the ability of antibody to inhibit haemagglutination ability and neutralize infectivity is not always observed (Kida et al., 1982). The determination of the precise mechanism of action of neutralizing antibody at the molecular level has only recently been addressed (Possee et al., 1982; section 9.2; section 12).

Within the view that antibody to the HA is mainly responsible for preventing infection is the undetermined relative importance of systemic or secretory immunoglobulins.

Fazekas de St. Groth (1950) concluded that the resistance in mice to influenza depended on the presence of antibody in the respiratory tract rather than in the serum. Further investigations in man and animal models (Review: Couch et al., 1969) seemed to firmly establish the primary role of secretory antibody in preventing influenza. The predominant immunoglobulin present in respiratory secretions in man is secretory IgA (Tomasi et al., 1965) and this form of IgA is only found in low concentrations in serum. IgA is synthesized in the local lymphoidal tissues as a dimer and appears as sIgA on the external surface after acquiring secretory component (Sc) during transport across the mucosal epithelium (Lamm, 1976; Nagura et al., 1979). Secretory IgA is resistant to proteolytic enzymes and its multivalence (associated with high avidity) may be especially relevant in neutralization of viruses. The local anamnestic IgA response is promptly recalled (Wright et al., 1983). All these properties would seem to make sIgA uniquely qualified to develop a local anamnestic response and Rossen et al., (1970) demonstrated that secretory IgA (sIgA) was responsible for the neutralizing activity of nasal secretions for influenza virus. However, this and subsequent studies that have demonstrated the importance of sIgA (Reviews: Rossen et al., 1971; Shvartsman Ya and Zykov, 1976; Couch and Kasel, 1983) have relied on artificially high intranasal doses of influenza for re-challenge in contrast to the low doses that lead to natural (re-)infection. While there is no question about the antiviral effects of sIgA

its presence in the respiratory secretions cannot always be correlated with protective role of secretory antibody. IgM and IgG are also found in respiratory secretions and antiviral activity has been observed in all immunoglobulins present (Ogra et al., 1975). These immunoglobulins secreted in the respiratory tract could have diffused from the systemic system or been synthesized locally (Rossen et al., 1971).

Couch et al., (1979) showed that the level of serum antibody in human volunteers related best to protection as there was an inconsistent detection of nasal neutralizing antibody. It was also shown (Couch et al., 1981) in human volunteer challenge studies that there was a better correlation between specific IgG in the respiratory secretions than with the sIgA. Potter and Oxford (1979) maintain that serum IgG is the most important determinant of resistance. Evidence is also available to suggest that protection against mucosal infection in asymptomatic patients with selective IgA deficiency may be effectively provided by the compensatory local production of IgM and IgG virus-specific antibodies (Ogra et al., 1974). However, in another human study with improved sensitivity for antibody detection, Murphy et al. (1982) demonstrated a solid (100%) correlation between protection and sIgA. At the same time IgG and IgM HA antibody were detected inconsistently in the respiratory secretions. Using a mouse model Liew et al. (1984) demonstrated that sIgA is the single most important factor contributing to protection against homotypic influenza infection, there was a



lack of correlation between protection and serum antibody.

On the basis of the profile of influenza pathogenicity (section 4) it is logical to assume that systemic and secretory immunoglobulins participate synergistically in specific immunity to influenza infection. Clearly sIgA contributes to immunity and may assume the primary role. However IgG in respiratory secretions, either produced locally or derived from the systemic circulation, is also an important mediator of resistance. A definitive study of the sIgA response establishing its relative importance and characteristics in protection to naturally occurring infection has yet to be carried out.

The immune response to the first infection by influenza virus can have a dominating influence on subsequent immune responses to antigenically related strains. Infection with a novel influenza strain can induce an antibody response that is mainly directed against the the strain responsible for the first infection at the expense of a response to the latest infection. This domination of the immune response to the original virus strain is called the "original antigenic sin" (Davenport et al., 1953). This has important implications in terms of immune status and susceptibility to influenza explaining the inefficiency of certain vaccines and paradoxical anti HA responses in man (Schild et al., 1977; Virelizier et al., 1979). Lamb et al. (1982) propose that the cross reactivity of the intermolecular help between Th cells and B cells could help to explain the

phenomenon. A strong Th signal might reactivate an antigenically "incorrect" B cell and plasma cell response whose secreted immunoglobulins cross-react only weakly with the newly presented HA.

It is apparent that cell-mediated responses do occur in man and contribute to resistance (McMichael et al., 1982). For influenza virus the protective effect of Tc cells against lethal doses in mice was demonstrated by adoptive transfer by Yap et al. (1978) and using a cloned murine Tc cell line by Lin and Askonas (1981). Infection generates Tc that do not discriminate between serologically distinct influenza viruses within a subtype (section 5.4). In humans natural influenza infection boosts T cell memory (McMichael et al., 1983b) but this is time-limited. McMichael et al. (1983b) state that human Tc cell memory has a half-life of 2-3 years after natural infection, whereas Ennis (1982) found that six months after vaccination with inactivated virus T cell memory was back to baseline levels. This could indicate that Tc offer a degree of (short-lived) heterotypic immunity. However, Armerding and Liehl (1981) achieved protection between heterotypic influenza strains in the presence of cyclosporin A which reduces immune responses without affecting antibody levels. It was suggested that this heterotypic immunity may be due to virus cross-reactive T helper cells inducing antibodies. In addition arguing against the role of Tc is the fact that some cloned Tc cell lines with strong cytotoxic activity were not found to provide protection *in vivo* (Taylor and

Askonas, 1983). Liew et al. (1984) have found that the transfer of a polyclonal Tc cell population with a combined strong cytotoxic activity did not reduce the titres of virus in the lungs of mice.

Further it was reported (McMichael et al., 1982) that mice treated with anti-IgM and produced no detectable HI antibody in any subclass recovered from infection and were immune to re-challenge, suggesting that T-cell response may have an important role. In contrast to immunoglobulin molecules that can be produced rapidly in enormous quantities following re-challenge, T cell numbers are restricted by the frequency of precursors and the doubling time of such cells (Burnet 1970; Doherty and Korngold, 1983). Studies in man and animal models show a clear correlation between antibody and resistance to infection. A decisive role for T cell immunity in protection on current evidence has not been clearly demonstrated, although in the recovery phase Tc cell activity could be crucial. Further investigations could include studies designed to detect the contribution of cellular immunity in the respiratory tract from local lymphoidal tissue (lymph nodes and lamina propria). Studies have demonstrated that cellular immunity in the respiratory tract can be produced independently of systemic immunity (Waldman and Henney, 1971). It has been reported that macrophage inhibitory factor (MIF) from sensitized lymphocytes is present in the bronchial washes of humans in response to influenza vaccine (Waldman et al. 1972). These locally sensitized lymphocytes

remain in close proximity to the respiratory tract and do not migrate in significant numbers into the systemic circulation.

## VIRUS NEUTRALIZATION BY IMMUNOGLOBULINS

### 6.1. The nature of the neutralization reaction

The attachment of neutralizing antibody to virus particles results in a loss of infectivity. Neutralization is mediated by antibodies of the IgG, IgM and IgA classes (Ogra et al., 1975). Some of the properties of these immunoglobulins are summarized in Table 6.1. The IgD and IgE classes have not yet been implicated in neutralization. The loss of infectivity of viruses due to neutralizing antibody alone in the absence of accessory factors (i.e. complement) can be due to several different mechanisms (Reviews: Mandel, 1979; Dimmock, 1984).

### 6.2. General properties of antibodies.

#### An Overview:

Immunoglobulins are multidomain glycoproteins that display a remarkably diverse range of binding specificities for discrete antigenic determinants or epitopes on the surface of molecules. The mammalian immune system is capable of expressing millions of different paratopes (antibody binding sites) from a relatively small number (a few hundred) genes. (Review: Kindt and Capra, 1984). The genetic mechanisms responsible for the diversity of

Table 6.1. Summary of the properties of immunoglobulins implicated as being involved in the immune response to virus in man \*.

	Immunoglobulin class			
	IgG	IgM	IgA (a)	sIgA
Property:				
Approx. Mol. wt.	150,000	900,000	160,000	385,000
Heavy ch.	gamma	mu	alpha	alpha
Structure	4 ch. unit	(4 ch. unit) <sub>5</sub> + J ch.	4 ch. unit	(4 ch. unit) <sub>2</sub> + J ch. + Sc
Valency	2	10	2	4
% total Ig in serum	80	6	13	neg.
Subclasses	1,2,3,4	1,2	1,2	1,2
Complement fixation	+ (b)	+	-	-

\* Not including IgD and IgE.

(a) Also present in very low concentrations in serum as a dimer polymerized by J chain only, total Mol. wt. 335,000.

(b) The IgG4 subclass of human IgG is not capable of activating complement.

neg = negligible; ch. = chain; Sc = secretory component.

n.b. concentration of immunoglobulin (Ig) may vary in individuals.

antibodies will not be discussed here except to say that the variable regions of different antibodies have different paratopes by virtue of germ-line gene selection and combination, and further by somatic recombination events in cell division and somatic point mutations in B cells. This creates a vast repertoire of paratopes each of which are able to give a stable union with an epitope of sufficient complementation.

Common to the immunoglobulins of vertebrates from sharks to the human is a four-chain structure consisting of two light (L) chains and two heavy (H) chains; however some immunoglobulins are polymers of the four chain monomeric unit (Reviews: Porter, 1973; Nisonoff et al., 1975; Burton, 1985). IgG has the basic four-chain immunoglobulin structure in the shape of a Y, as illustrated in Figure 6.1. Each L chain is linked to an H chain by strong noncovalent forces and, in most immunoglobulins, by one or more interchain disulphide bond. Similar forces are involved in holding together pairs of H chains. Each H chain and each L chain consists of a variable (V) and constant (C) part. The V regions are responsible for the diversity of antibody specificities. The C regions are invariable for a given class of H chain or type of L chain, except for minor genetic polymorphisms. There are five classes of H chain which define five classes of immunoglobulin: IgM, IgA, IgG, IgD, and IgE. The C region of the H chain defines the class of the immunoglobulin. Mammals possess two types of L chain called kappa and lambda, which can combine with H chains of any class. An antigen binding

site is made up of amino acids from one H chain and one C chain. Thus, the four-chain monomer possesses two combining sites. Within each V region are three clusters of hypervariable amino acids that contribute directly to the formation of the antigen combining site. Both H chains and L chains are made up of domains, each domain containing 110-120 amino acid residues. Each domain also has an intra-chain disulphide bond, spanning 55-65 amino acid residues. Each L chain has two domains (V and C). Each H chain has one V domain and three or four C domains depending on its class. A diagram of the structure of one of the two sub-classes of rabbit IgG is given in Figure 6.1, illustrating some of the points made above.

Antigenicity of proteins is determined by the accessibility, hydrophobicity and mobility of a particular region (Review: Van Regenmortel (1986)). The importance of each feature can only be defined with reference to a particular antibody-antigen reaction. The mobility component is undeniably an important factor (Review: Tainer et al., 1985) involved in a region of a protein adopting a conformation suitable for recognition by antibody, i.e. to an epitope which is complimentary to the paratope.

There are continuous epitopes, defined as a linear sequence of amino acid residues, or discontinuous (or assembled) epitopes, made up of residues that are not contiguous in sequence but are brought together at the protein surface by the folding of one or more polypeptide chains. When monoclonal antibodies directed to



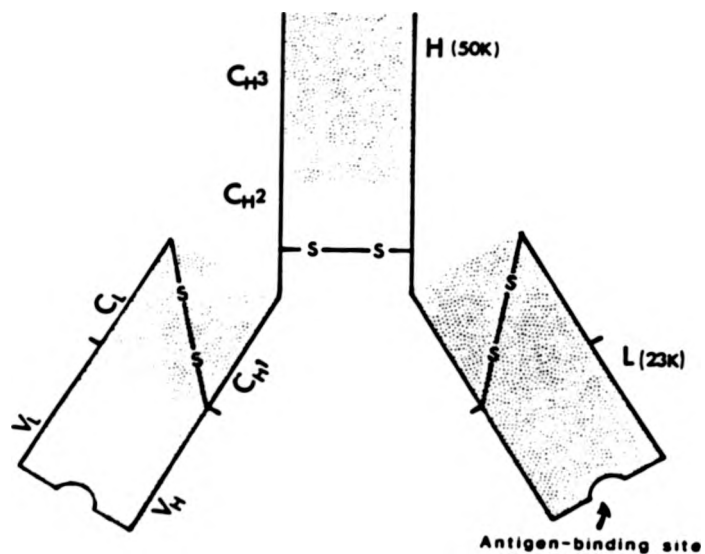
whole proteins are used to locate the position of epitopes mainly discontinuous epitopes are revealed (Benjamin et al., 1984). Few monoclonal antibodies are produced that recognize linear fragments of the antigen. Since monoclonal antibodies are selected for high affinity binding the pattern of epitope-paratope interaction revealed by them may not be representative of the reactions that occur with low affinity antibodies.

The properties of IgG, IgM, IgA and sIgA are summarized briefly below. Factors affecting neutralization mediated by antibody are addressed in this thesis and encompass class of immunoglobulin, valency, and quantitative aspects.

#### 6.2.1. IgG

IgG is the major circulatory and anamnestic immunoglobulin in mammals. It has the basic four-chain immunoglobulin structure (Figure 6.1) in the shape of a "Y". Human IgG occurs as four major sub-classes: IgG1, IgG2, IgG3, and IgG4 which vary in concentration in the serum. The normal order of relative concentration of IgG subclasses in humans is IgG1 > IgG2 > IgG3 > IgG4. There can however be individual variation in the values. The subclasses differ in the C region of the H chain and consequently in the effector functions mediated via these regions such as complement fixation, placental passage and binding to phagocytes (Review: Burton, 1985). In the mouse there are four

Figure 6.1. Diagram of the structure of the rabbit IgG molecule. In an individual molecule the two H chains are identical to one another, as are the two L chains. The shaded areas represent regions that are held together by non-covalent bonds.



sub-classes of IgG (IgG1, IgG2a, IgG2b, and IgG3) and in the rabbit two (IgG and IgG1) although IgG1 is present in very low concentrations.

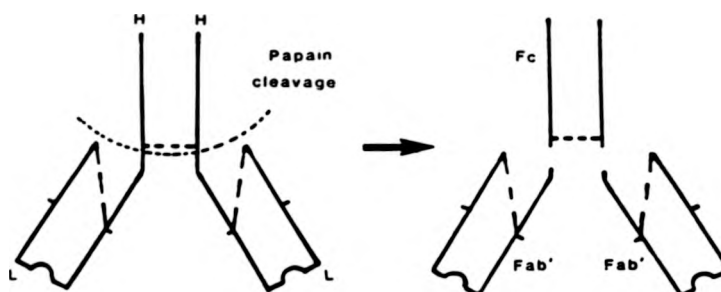
Native IgG molecules can be cleaved into large fragments by certain proteolytic enzymes. Papain cleaves IgG on the N-terminal side of the disulphide bridge that joins the two H chains to yield two monovalent Fab' fragments and a Fc fragment (Figure 6.2.1). A Fab' (ab for antigen binding) fragment contains a complete L chain and the VH and CH1 regions. Being monovalent, Fab fragments cannot form crosslinked networks and are therefore incapable of forming aggregates. The Fc (crystalizable) fragment contains the C-terminal halves of both H chains. Pepsin cleaves IgG on the C-terminal side of the disulphide bridge that joins the two H chains removing a partially degraded Fc portion and a F(ab')<sub>2</sub> fragment that retains bivalency (Figure 6.2.2)

#### 6.2.2. IgM

A diagrammatic representation of the structure of a human IgM molecule is shown in Figure 6.3. IgM is the earliest immunoglobulin to appear in the serum of a mammal after stimulation with an antigen but is progressively replaced by IgG during the response. In most species IgM has a pentameric structure (five four chain monomeric units) and a valency of 10. Because identical H and L-chain pairs form combining sites, the specificities of the 10 combining sites in an individual molecule

Figures 6.2.1. and 6.2.2. Location of cleavage sites in mouse IgG sensitive to enzymes papain (Figure 6.2.1.) and pepsin (6.2.2).

Figure 6.2.1. The Fab' fragments produced from IgG by papain are monovalent. The Fc region contains amino acids derived only from the constant regions of the heavy chains. Each Fab' fragment contains one complete L chain and approximately half of an H chain.



**Figure 6.2.2.** The  $F(ab')_2$  fragment produced by pepsin is bivalent; the  $F_c$  segment is partially degraded. If the disulphide bond between the two heavy chains is subsequently reduced (i.e. with cysteine), two monovalent  $Fab'$  fragments are released.

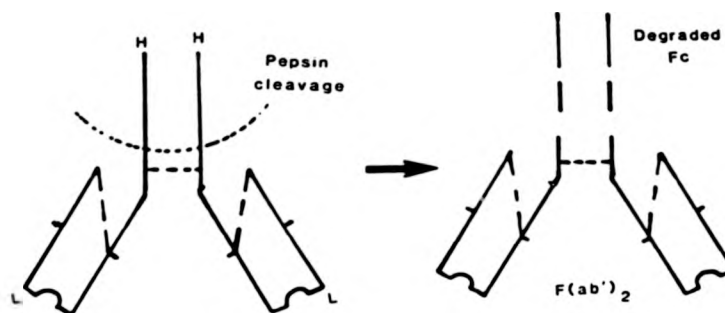
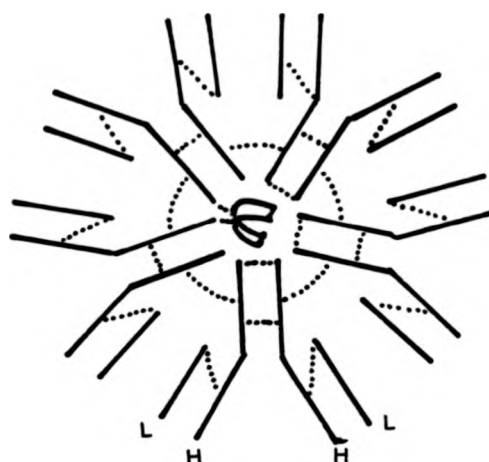


Figure 6.3. Diagram of the structure of the human IgM molecule. The dashed lines represent the intrachain disulphide bonds.



 = J chain

are identical. In the pentameric structure each four chain unit is joined to an adjacent unit by a single disulphide bond between two  $\mu$  chains. Mild reduction at neutral pH (Nisonoff et al., 1975) yields 5 four-chain units (monomeric IgM). Trypsin or papain can be used to prepare  $F_{\mu}$  and  $Fab'_{\mu}$  fragments of IgM (Plaut and Tomasi, 1970) and pepsin can be used to produce  $F(ab'_{\mu})_2$  fragments (Dorrington and Tanford, 1970). Pentameric IgM molecules each contain a J chain (MW 15,000) which appears to be essential for initiating polymerization through disulphide bonds of the four chain units of IgM (and dimeric IgA). IgM is a potent activator of the classical complement pathway since there are, on a single molecule, 5 Fc regions that have attachment sites for Clq.

#### 6.2.3. IgA and sIgA

The majority of serum IgA in humans is monomeric and consists of the basic four chain unit. sIgA is the predominant immunoglobulin on the mucosal surfaces of the body of most mammals and is therefore present in the first line of defence against infection of the respiratory, intestinal and urogenital tracts (Reviews: Ogra et al., 1984; Dhar and Ogra, 1985). sIgA (Figure 6.4) is a dimer of the basic four chain unit structure linked by a J chain and the secretory component (Sc). Sc is acquired when the molecule is transferred across the mucosal epithelium. Dimeric IgA may also be present in negligible amounts in the serum as sIgA or as a dimer polymerized by the J chain

Figure 6.4. Diagram of the structure of the human secretory IgA molecule. Secretory component (Sc) is joined to the alpha chains by strong non covalent bonds as well as by disulphide bonds. A J chain is also contained in the molecule.

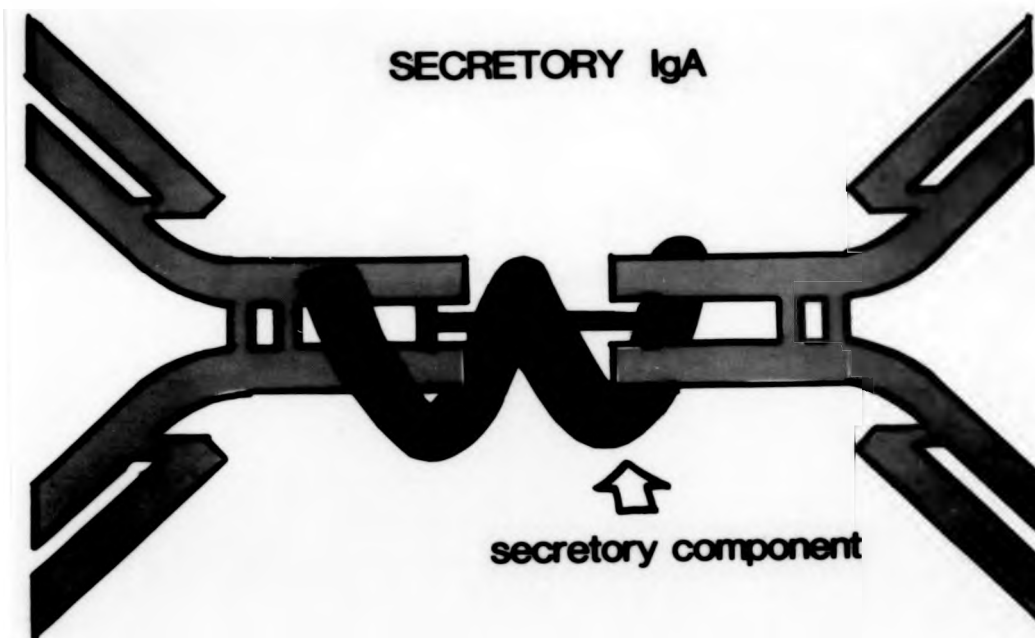




Figure 6.4. Diagram of the structure of the human secretory IgA molecule. Secretory component (Sc) is joined to the alpha chains by strong non covalent bonds as well as by disulphide bonds. A J chain is also contained in the molecule.

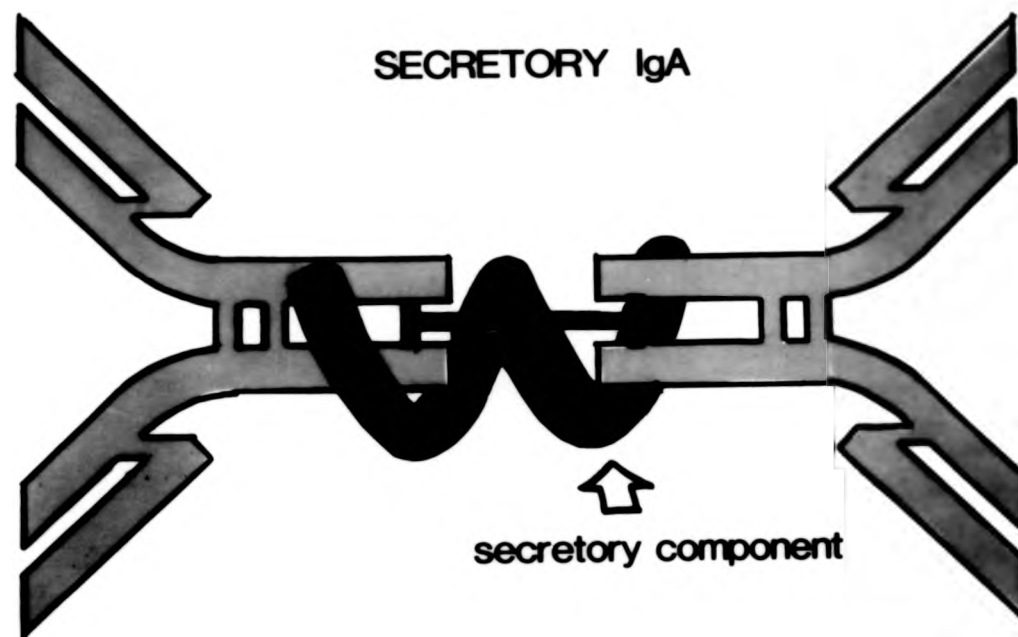
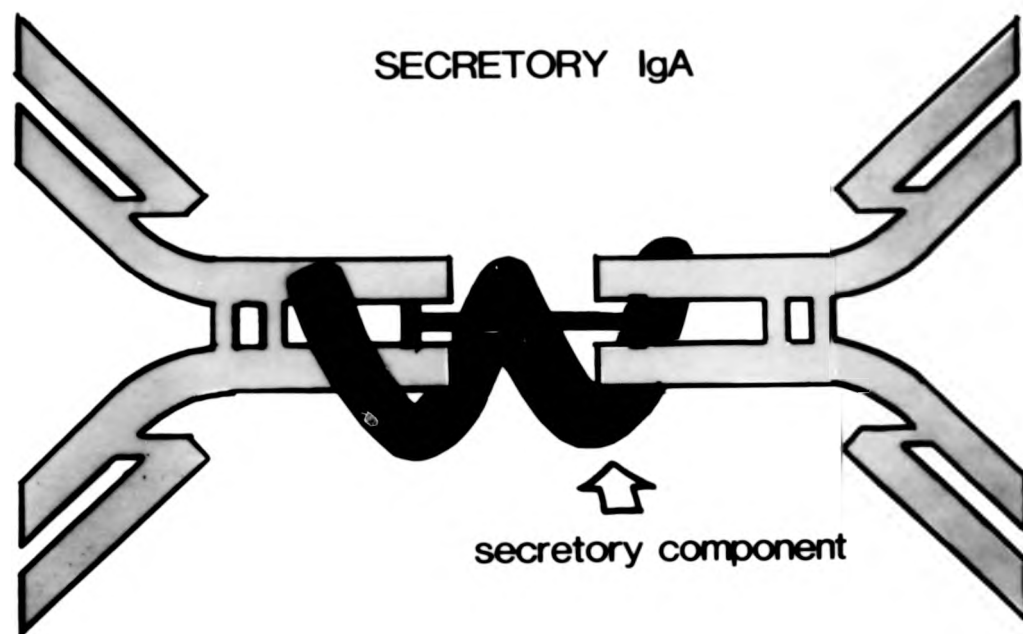


Figure 6.4. Diagram of the structure of the human secretory IgA molecule. Secretory component (Sc) is joined to the alpha chains by strong non covalent bonds as well as by disulphide bonds. A J chain is also contained in the molecule.



only. Higher polymers of IgA can occur notably the tetrameric sIgA that is found in man (Tomasi, 1976).

The origin of the sIgA in secretions can be attributed partly to local production of IgA in plasma cells located in the mucosal lamina propria, which underlies the luminal surface epithelium (Reviews: Lamm, 1976; Borsch, 1984). Investigations of the origin of these cells has lead to the concept of a common mucosal immune system, whereby cells that are stimulated to produce IgA antibody to antigens at one mucosal site subsequently migrate to and populate distant mucosal sites (Bienenstock and Befus, 1980). Serum polymeric IgA may also gain access to mucosal sites and selective transport of serum derived IgA can occur and represents a connection between the serum and external secretions (Hall et al., 1979; Review: Hall and Andrew, 1980; Sullivan and Arrowsmith, 1984), however this is dependent on the availability of Sc (Sheldrake et al., 1984).

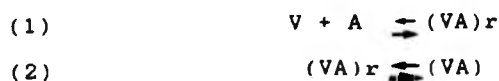
It should be noted that the distribution of immunoglobulins in secretions differs in different mammalian species (Tomasi, 1976). In humans and rodents sIgA is the predominant class of immunoglobulin; however significant amounts of other classes are also found and these can contribute to the mucosal immune response. In sheep IgG is the predominant immunoglobulin in secretions and sIgM (IgM + Sc) is the predominant immunoglobulin in rabbit external secretions.

Despite the obvious differences between the subunit and polypeptide chain assembly of sIgA and serum (monomeric) IgA, at present there is no evidence of any difference in the primary structure of either the H $\alpha$  chains or L chains.

sIgA is the most resistant of all immunoglobulins to proteolytic digestion, reflecting the harsh environmental conditions (pH, ionic strength, detergent, proteases) in which sIgA must function. Pathogenic bacteria such as *Streptococcus pneumoniae* and *Neisseria gonorrhoeae* produce IgA<sub>1</sub> proteases that have exquisite specificities for IgA of the IgA<sub>1</sub> subclass of humans (Review: Plaut, 1983). Fab'  $\alpha$  and F(ab'  $\alpha$ )<sub>2</sub> fragments can be obtained however from secretory and monomeric IgA by several methods and monomeric IgA can be obtained from sIgA by partial reduction (Reviews of methods: Tomasi, 1976; Johnstone and Thorpe, 1985)

#### 7.1. Significance of valency and flexibility of immunoglobulins in viral neutralization.

There is an equilibrium between dissociation and reformation of antibody-virus complex (Review: Mandel, 1979). Virus (V) reacts with antibody (A) in two steps:



(VA)<sub>r</sub> represents virions complexed with antibody in a freely reversible way; (VA) represents virions complexed with antibodies in an almost irreversible way.

The equilibration of (1) is reached rapidly and is not temperature dependent, whereas the equilibration of (2) is slow at 0° C but at 37° C proceeds rapidly so that in minutes most of the antibody virion complexes formed are stable. Although increase in temperature favours the rate of reaction, a decrease in temperature favours the formation of the complex because of an accompanying decrease in enthalpy. Szweczuk and Mukkur (1977) have suggested that the relative contributions of the changes in enthalpy and entropy to the forward reaction vary with temperature. At low temperature the enthalpy change is the principal driving force, whereas at high temperature (37°C) the change in entropy is more significant. The degree of reversibility diminishes the longer the reaction is allowed to proceed.

Affinity is simply the strength with which the antibody binds to its antigenic determinant. The avidity of an antibody relates to its valency. When a single IgG molecule binds to two sites on a single influenza virus HA spike (Figure 7.1.1) or adjacent HA spikes on the same virus particle (Figure 7.1.2), the stability of the complex increases by several orders of magnitude. The probability of simultaneous dissociation of the paratopes is small. Fab' fragments having one paratope produced

**Figure 7.1.1.** Diagram of IgG with both binding sites attached to a single HA spike (trimer) of an influenza virus particle. The antigenic regions are shaded in pink, the location of amino acid residue 266 in the VAS is shaded blue.

Diagram drawn to scale (1 cm = 2.4nm):

HA structure adapted from Wiley et al., 1981; Wilson et al., 1981; Rogers et al., 1983. IgG structure determined from Werner et al., 1972; Nisonoff et al., 1975; Silverton et al., 1977; Deisenhofer, 1981; Marquart and Deisenhofer, 1982. N.B. The Fc region of IgA has approximately the same dimensions as that of IgG.

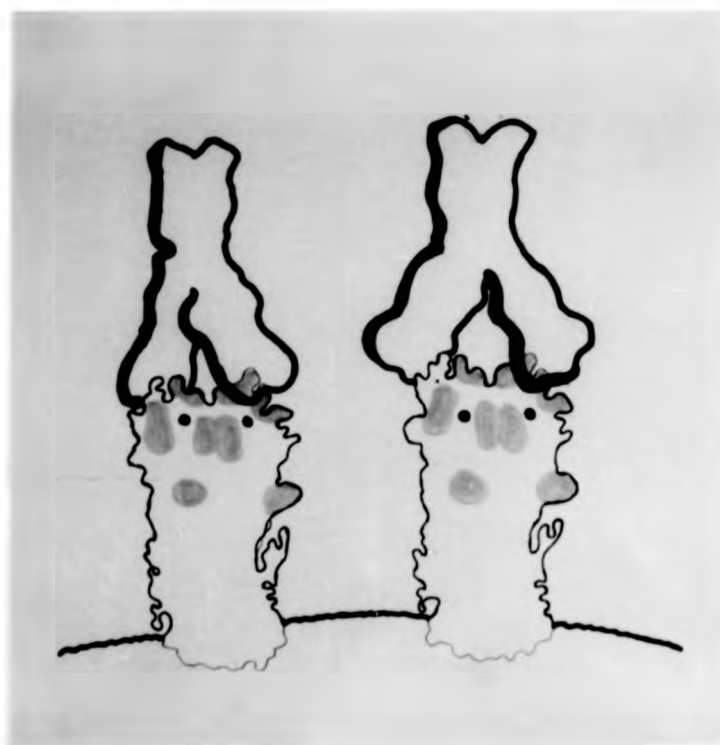


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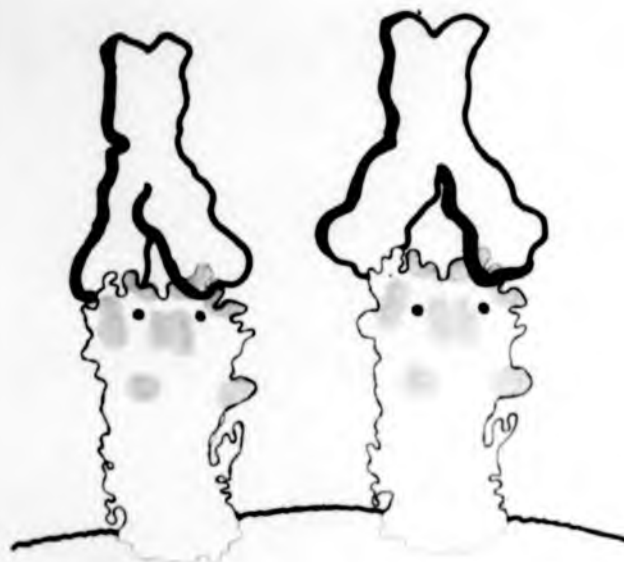
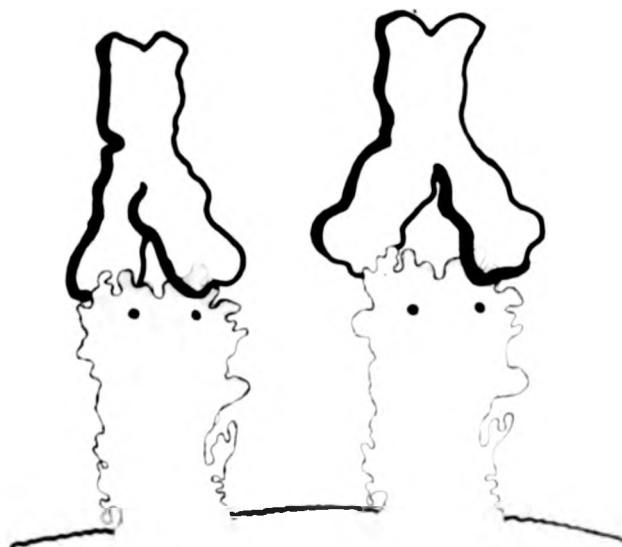


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Diagram drawn to scale (1 cm = 2.4nm):

HA structure adapted from Wiley et al., 1981; Wilson et al., 1981; Rogers et al., 1983. IgG structure determined from Werner et al., 1972; Nisonoff et al., 1975; Silvertown et al., 1977; Deisenhofer, 1981; Marquart and Deisenhofer, 1982. N.B. The Fc region of IgA has approximately the same dimensions as that of IgG.





**Figure 7.1.1. Diagram of IgG with both binding sites attached to a single HA spike (trimer) of an influenza virus particle. The antigenic regions are shaded in pink, the location of amino acid residue 266 in the VAS is shaded blue.**

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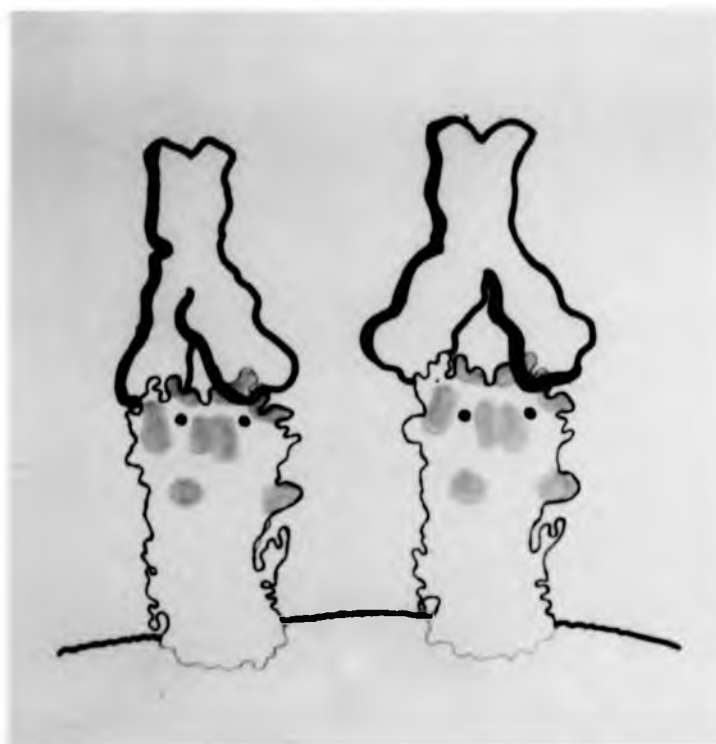


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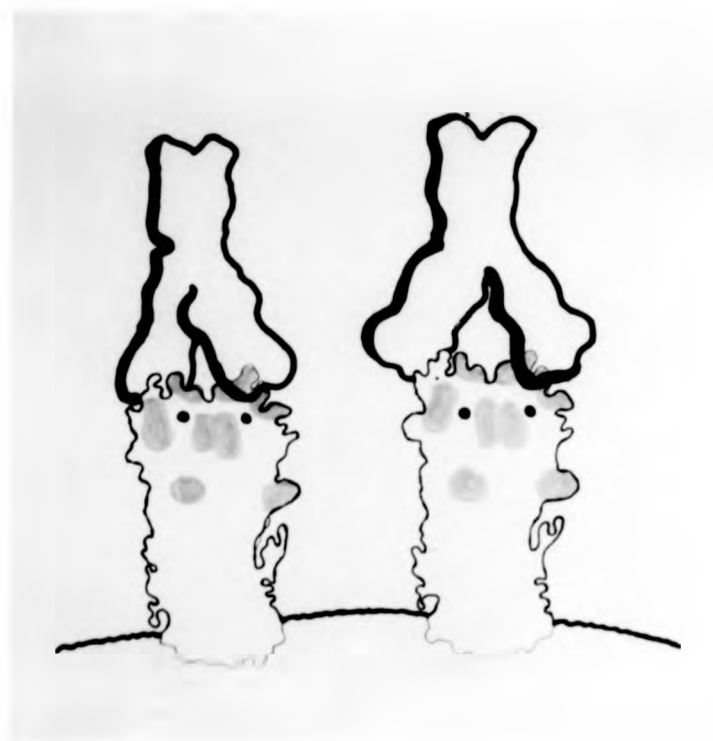
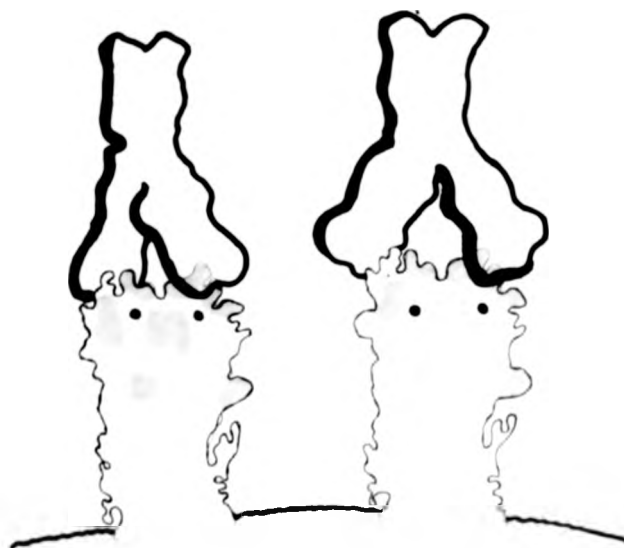


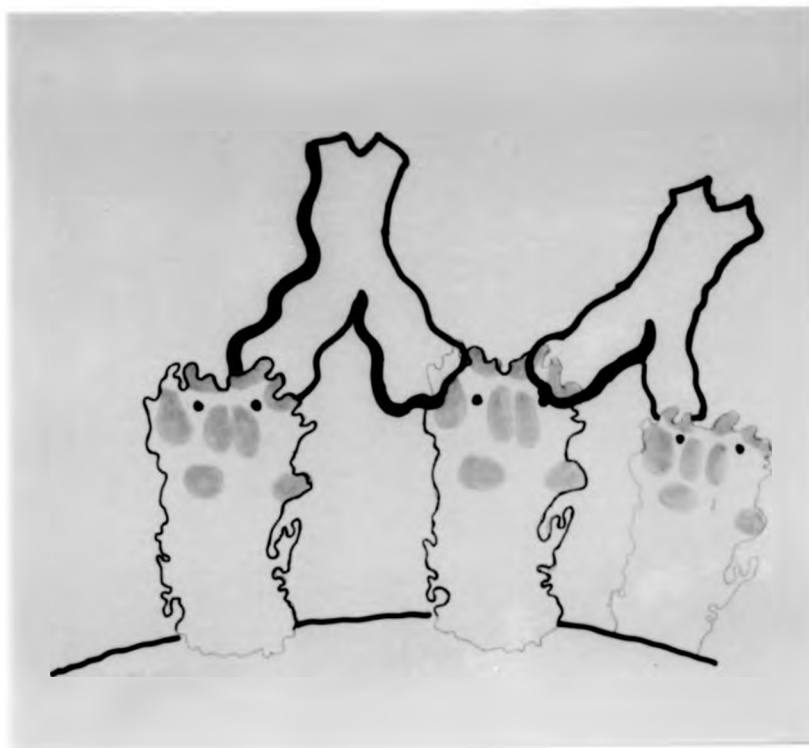
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Diagram drawn to scale (1 cm = 2.4nm):

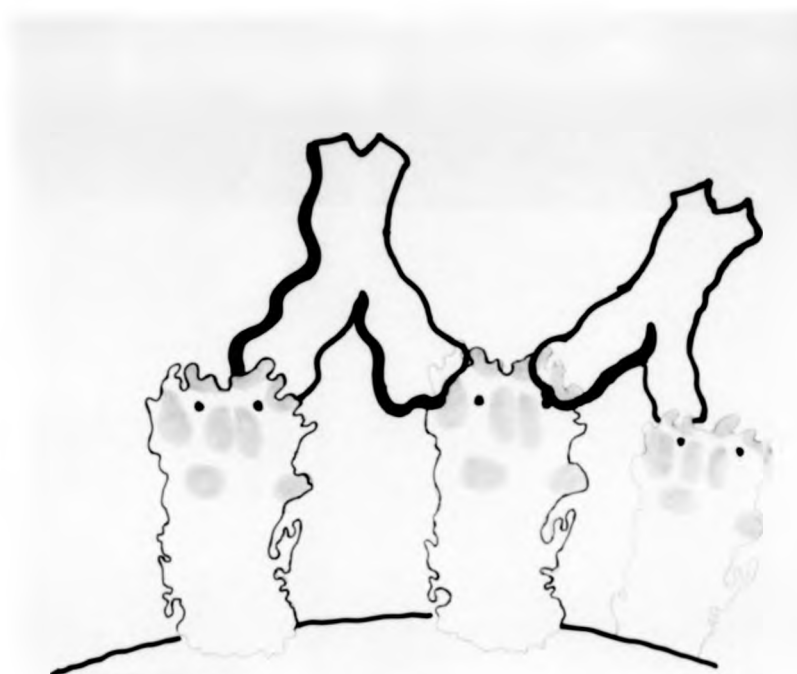
HA structure adapted from Wiley et al., 1981; Wilson et al., 1981; Rogers et al., 1983. IgG structure determined from Werner et al., 1972; Nisonoff et al., 1975; Silverton et al., 1977; Deisenhofer, 1981; Marquart and Deisenhofer, 1982. N.B. The Fc region of IgA has approximately the same dimensions as that of IgG.



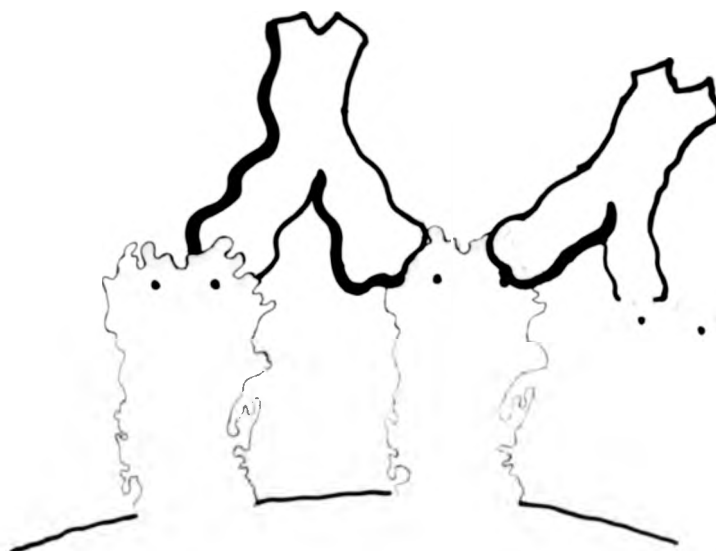
**Figure 7.1.2.** Diagram of IgG binding to two adjacent HA spikes of an influenza virus particle. It should be noted that anti-HA IgG molecules are also capable of cross-linking virus particles. Diagram drawn to scale (see Figure 7.1.1).



**Figure 7.1.2.** Diagram of IgG binding to two adjacent HA spikes of an influenza virus particle. It should be noted that anti-HA IgG molecules are also capable of cross-linking virus particles. Diagram drawn to scale (see Figure 7.1.1).



**Figure 7.1.2.** Diagram of IgG binding to two adjacent HA spikes of an influenza virus particle. It should be noted that anti-HA IgG molecules are also capable of cross-linking virus particles. Diagram drawn to scale (see Figure 7.1.1).



by papain digestion of IgG form much less stable complexes. Their stability depends on the stability of the native immunoglobulin, i.e. very high affinity IgG could give effectively stable binding by Fab'. Important properties of sIgA (Figure 6.4) and IgM (Figure 6.3) are their multivalency (4 and 10 binding sites respectively) resulting in high avidity for antigen, even if the affinity (of the four chain unit) is low. This may be especially relevant in the neutralization of virus.

A general theory for antibody action (Metzger, 1974) was that cross-linking of antigens is required for physiological effects, i.e. mechanical cross-linking by  $F(ab')_2$  arms is the minimal requirement for antibody action. However, observations of the actions of Fab' fragments derived from anti-lymphocyte and anti-thymocyte IgGs (Van Wauve et al., 1980; Guillet et al., 1983) show that bivalent cross-linking is not always a necessary requirement for complete function of antibody. Much earlier Lafferty (1963b) showed that monovalent Fab' fragments neutralize influenza virus with comparable efficiency to antibody but that neutralization was reversed on dilution. No cross-linking such as that achieved with multivalent antibody was necessary. Although Fab' fragments derived from polyclonal IgG neutralized poliovirus (Vogt et al., 1964; Keller, 1966) recent studies with Fab' fragments derived from monoclonal IgG (Icenogle et al., 1983; Emini et al., 1983a) have generally suggested that cross-linking is required for neutralization of poliovirus (section 9.2.2). The mechanisms of neutralization of influenza virus by Fab' and

F(ab')<sub>2</sub> fragments derived from monoclonal IgG are investigated in this thesis.

The flexibility of immunoglobulin molecules is an important consideration in the interaction of antibody with viral antigens. Complement activation by antibody is dependent on the flexibility of the antibody (Review: Feinstein et al., 1986). A flexible stretch of amino acids between the CH1 and CH2 domains known as the hinge region (Huber et al., 1976) allows an IgG molecule to assume various "Y" configurations. The pivotal area about which Fab-Fc movement occurs or the degree of movement within a IgG molecule that occurs in a given antibody-antigen reaction is not known with any certainty (Review: Burton, 1985). Valentine and Green (1967) in electron microscopic (EM) studies determined that the Fab' arms of rabbit IgG binding to hapten could assume angles of 10 to 180° relative to each other. The observations in solution with haptens indicate that "T" shaped configurations (180° separation) can bridge haptens at least 9nm and up to approximately 15nm apart (Werner et al., 1972). Wrigley (1979) showed that IgG binding to isolated influenza A BrHA could bridge molecules with an extended T shaped configuration. IgG molecules were seen by Lafferty and Oertelis (1963) to accommodate antigenic sites up to 12nm apart on adjacent HAs of intact influenza virus (Figure 7.1.2) or with both Fab' arms attached to antigenic sites on the same HA in a classic Y shaped configuration (Figure 7.1.1). Extended IgG molecules were seen also to link two influenza virus particles.



Nanosecond fluorescence measurements have been interpreted to indicate segmented flexibility and motion of the Fab' arms about the hinge region of IgG in solution (Hansen et al., 1981). This motion is unaffected by binding of Clq or protein A to IgG. Wrigley et al. (1983) obtained evidence by EM studies for the ability of mouse IgG<sub>1</sub> to twist around its long axis and for C-V region bending in Fab' arms in the interaction of monoclonal antibodies with BrHA molecules in solution. Flexibility is known to vary with IgG subclass in the mouse (Oi et al., 1984) and inflexibility in the hinge region has been associated with loss in Fc effector functions such as Clq binding (Dorrington and Klein, 1982).

EM studies have shown that an uncomplexed IgM molecule adopts a star-shaped planar conformation (Chesebro et al., 1968; Feinstein et al., 1971) (Figure 7.1.3). IgM has been visualized as binding to antigen in various configurations and assumes a variety of shapes (Feinstein et al., 1971) notably with a "spider-like" docking orientation, i.e. with all 10 Fab'<sub>mu</sub> arms attached to the antigen (Figure 7.1.3). The span of the IgM molecule in a "spider like" conformation is approximately 25 nm (Feinstein et al., 1986). This configuration of IgM is the best activator of complement (Feinstein et al., 1986). The high valency of IgM ensures that cross-linking of virus particles is possible as demonstrated by Brown and Smale (1970) with IgM cross-linking foot and mouth disease virus. EM examination of

side views of IgM cross-linking flagella support the model of IgM having each  $F(ab'\mu)_2$  unit rotated through  $90^\circ$  about its two fold axis of rotation so that each pair of  $F(ab'\mu)_2$  arms lies in right angles to the main disc (Feinstein et al., 1986). The degree of flexibility of the hinge region of IgG is greater than that of IgM (Review: Feinstein et al., 1986). However, it should be borne in mind that the five-fold higher valency of the IgM molecule may more than compensate for its lower flexibility.

Relatively little information is available concerning the 3 dimensional structure of sIgA. The predominant form in free solution is of the sIgA molecule fully extended (Tomasi, 1976, Hall and Andrew, 1980) (Figure 7.1.3). IgA molecules possess a hinge region similar to that of IgG and sIgA molecules have been observed (Tomasi, 1976) with bending at a point in the middle of the molecule so that four chain units are able to flex  $60-120^\circ$  relative to each other. This probably occurs at the joining position of the two monomers as dimeric IgA in the serum (without Sc) shows a tendency to bend at a point where the Fc regions are in close proximity. An electron micrograph (Hall and Andrew, 1980) shows one sIgA molecule attached to the flagellum of the bacterium *Salmonella paratyphi*. The molecule was orientated in the double Y extended conformation parallel to the antigen so that only one Fab'alpha arm of each four chain unit was binding to the flagellum. This docking orientation if common would not take advantage of the valency of the sIgA molecule but the degree of flexibility suggests that sIgA may indeed bind with all four

Figure 7.1.3. Diagram of some of the possible conformations of sIgA and IgM that could occur on binding to viral antigen.

Diagrams are adapted from 3-D models proposed by Burton (1986) and Pumphrey (1986).

sIgA in: (a) double Y conformation bound to viral antigen through only one four chain immunoglobulin unit, (b) in double Y conformation but bound to viral antigen via only one Fab'alpha arm of each four chain immunoglobulin unit, and (c) binding in hair-pin conformation with both four chain immunoglobulin units attached to viral antigen.

sIgA



a



b



c

IgM



d



e



f

IgM in: (d) a "star-like" planar conformation only one four chain immunoglobulin unit attached to viral antigen, (e) an intermediate conformation attached via three four chain immunoglobulin units, and (f) a "spider-like" conformation adopted after making multiple attachments to viral antigen (span = 25nm).

paratopes to repeating epitopes on antigens (Figure 7.1.3).

### 8.1. Attributes of the neutralization reaction.

To achieve neutralization an antibody has to bind to a site that is involved in the infectious process of the virus, i.e. a critical site (Daniels, 1975). Neutralization results from the binding of a number of molecules of immunoglobulin to one or more critical site(s) on a virus particle leading to loss of infectivity.

In order to neutralize influenza virus it is evident that antibody has to bind to not only to the HA but to certain specific regions on it (section 2.2; Figure 2.1.1). There are antibodies that bind to the HA of influenza but do not neutralize (Breschkin et al., 1981) presumably because they are directed to non-critical regions on the HA molecule. Binding to non-critical sites does not lead to neutralization. However, binding to non-critical sites may under certain conditions lead to "extrinsic" neutralization for example via the binding of complement (Haukenes, 1977). Laver and Webster (1966) found that antiserum to uninfected chick endodermal cells of the chorioallantoic membrane (CAM) inhibited the HA activity of influenza virus grown in chick embryos. They concluded that the presence of host antigen in the virus envelope (presumably carbohydrate) was responsible for this inhibition, probably due to steric hindrance of HA. Antibody binding to non-critical sites may because of topological disposition sterically interfere with a crucial function, for example attachment.

With appropriate concentrations of antibody and antigenic

sites the binding of antibody to non-critical regions could lead to secondary interactions such as aggregation. This is termed pseudoneutralization, where, although the individual viruses are themselves neutralized each multimeric aggregate registers as one infectious unit. Anti-NA does not neutralize influenza virus (Laver and Webster, 1966), but Compans et al. (1969) found that anti-NA could reduce the HA activity of an influenza virus population. It was concluded that this pseudoneutralization of HA activity was due to aggregation since ultrasonic vibration increased HA activity to 50% of control levels.

It should be noted that loss of infectivity may also be mediated by another component. For example complement may be required to inactivate the infectivity of an infectious virus-antibody complex. Although the mechanisms by which complement, together with immunoglobulin induces viral inactivation has not been addressed in this thesis, complement activation is an important facet of immunity (Review: Lachmann, 1985). The induction of viral neutralization by antibody and complement can occur in a number of different ways. For example, antibody may function together with complement to lyse enveloped viruses (Haukenes, 1977) aggregate (Oldstone et al., 1974) or smother virus in a coating of antibody and complement so as to block attachment of virus to the host cell (Berry and Almeida, 1968; Linscott and Levinson, 1969). In some virus infections the first antibodies that are formed are of low avidity and at low titre and depend on complement for neutralization (Lachmann,

1985). The majority of anti-HA IgG (detectable only by ELISA) from human sera to influenza A/WS/33 (H1N1) did not neutralize virus in direct neutralization assays (Beebe et al., 1983), but did so in conjunction with the first four reacting components of the classical complement pathway. Complement was activated leading to the deposition of C3 and C4 on the viral envelope. In addition complement can recognize and inactivate certain viruses directly (Welsh et al., 1976) in the absence of immunoglobulin.

The use of proper controls, defined conditions and monoclonal antibodies can largely exclude extrinsic phenomena from studies. The following discussion will deal largely with primary neutralization, pertinent to research described in this thesis.

It is now apparent that different viruses are neutralized by different mechanisms and that neutralization is further dependent on the host cell (Review: Dimmock, 1984). Available evidence suggests that any of the early events in virus replication may be interfered with by neutralizing antibody. These can be broadly categorized:

1. A critical viral function is negated when immunoglobulin molecules bind to sufficient critical sites to prevent the function dependent on that site. For example, the blocking of attachment of reovirus HAs to CRUs by the steric hindrance of IgG to reovirus HA (Lee et

al., 1981).

2. Virus neutralization is an active process and the interaction between virus and antibody leads to physiochemical alteration in the virus or conformational changes within the virion structure that modify the characteristics of a critical virus-cell interaction. Antibodies can induce conformational changes in virus proteins and these may be concerned with the neutralization process. A consequence could be the aberration or inhibition of an essential stage (or stages) in the viral replication cycle subsequent to attachment.

#### 8.1.1 Quantitative aspects of the antibody-virus interaction.

A crucial aspect of viral neutralization is the lack of an adequate explanation to reconcile single-hit inactivation kinetics (one antibody is sufficient to cause neutralization) with the multiligand nature of a virus particle.

The kinetics of the neutralization reaction can be used to examine these events. In kinetic experiments antibody is mixed with virus, a portion of the mixture removed and assayed for infectivity. Mathematically, the number of target sites that must be hit for inactivation of the virion is expressed by:

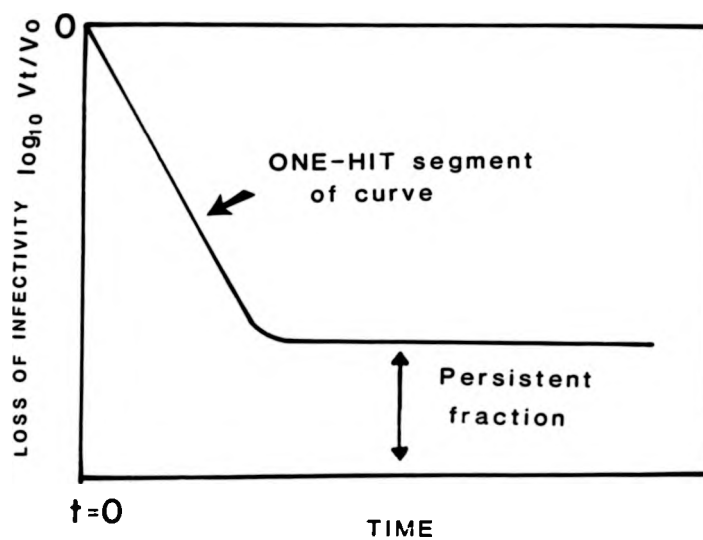
$$(1) \quad V_t/V_o = 1 - (1 - e^{-kt/D})^n$$



where:  $V_t$  = concentration of virus at time  $t$ ;  $V_0$  = concentration of virus at time 0;  $D$  = dilution factor;  $n$  = number of critical targets;  $k$  = reaction rate constant.

When  $n = 1$  the kinetics of neutralization of a virus can be graphically represented as an exponential decline in viral infectivity as a function of time. This is preceded by an immediate drop in viral infectivity. When  $n > 1$  there is an exponential decline, but only after a delay which is proportional to the value of  $n$ . From the studies with a variety of animal viruses (e.g. Dulbecco et al., 1956; Rubin and Franklin, 1957; Mandel, 1961; Thormar, 1963; Granoff, 1965; Phillipson, 1966; Review: Mandel, 1979) the most common type of survival curve is shown in Figure 8.1. Neutralization occurs immediately and proceeds at a constant rate for a set period of time. A decrease in the rate of neutralization then takes place and a persistent fraction results, which resists further neutralization. Based on the absence of a lag period in the first part of the the survival curve it is considered that the interaction of one molecule of antibody is sufficient to cause neutralization, i.e. a one-hit reaction. Mandel (1976) found that a transition in the isoelectric point of poliovirus from pH 7.0 to pH 4.5 occurred when virus was neutralized by antibody (section 9.2.2). Transition was an all or nothing phenomenon correlating directly to neutralization. It was considered that the neutralization of poliovirus was one hit

Figure 8.1. Kinetics of the neutralization of a typical virus. The loss in viral survival ( $V_t/V_0$ ) is graphed against the time elapsed after the addition of antibody ( $t=0$ ) (Adapted from Daniels, 1975).



because the transition of isoelectric point occurred in the presence of minimal concentrations of antibody and intermediate states were not observed.

The interpretation of the one hit curve has been called into question by kinetic experiments that have shown an initial lag in the rate of neutralization which suggests a multi-hit mechanism (Burnet et al., 1937; Lafferty, 1963a,b; Phillipson, 1966; Review: Della-Porta and Westaway, 1977). The delay implies that more than one antibody molecule must bind to neutralize the virion. Kinetic studies with low concentrations of antibody and at low temperature (4°C) indicated that the neutralization of influenza virus (Lafferty, 1963a,b) could be multi-hit. A lag was also seen using IgM antibody to poliovirus (Phillipson, 1966). The phenomenon of enhancement of Sindbis and West Nile viruses were utilized to demonstrate that more than one antibody was required for neutralization (Chanas et al., 1982; Peiris et al., 1982). Neutralizing antibody activity of monoclonal IgG was totally removed by dilution, but the presence of monoclonal IgG on the surface of virus particles could be shown by the enhancement of virus infectivity when virus particles treated with monoclonal IgG were incubated with cells bearing Fc receptors.

The reaction between antibodies and haptens is very rapid occurring in less than 3 seconds (Pecht et al., 1972). It has therefore been argued that the antibody-antigen reactions in

virus neutralization are also extremely rapid processes and the methods used for rate measurements do not indicate true rates. Thus, where there is no apparent lag phase in viral neutralization and a single-hit mechanism appears to operate, this may be due to the rapidity of the neutralization reaction thus making it impossible to accurately measure the reaction over the first few seconds. Ashe and Notkins (1967) with HSV and Hahon (1970) with Venezuelan equine encephalomyelitis virus (VEEV) established that very early on before any neutralization occurred antibody attached to the particle. Infectious virus-antibody complexes could be neutralized by anti-IgG containing serum. This further suggested that neutralization required more than one antibody to bind to the virus. Neutralization may be best described as sequential reaction:

Virus + antibody  $\rightarrow$  Infectious virus-antibody complex (V-ab)

V-ab + more identical antibody  $\rightarrow$  Neutralized virus.

If it is maintained that one (or at best a few) molecule(s) of antibody neutralize an infectious virus particle then the interpretation that neutralization occurs by the prevention of attachment of virus to a susceptible cell is brought into question. The surface of most animal viruses is composed of identical virus attachment protein (VAP) subunits each one of which carry virus attachment sites (VAS) which have the potential capability to bind to a cellular receptor unit (CRU). For example, it has been determined that influenza type A virus

particles have between 700 and 1200 spikes, 4 in 5 of which are HA (section 1.2); each HA spike is a trimer composed of three HA monomers each bearing a VAS (section 2.2). Therefore a conservative estimate of the number of sites available on an influenza particle to bind to a CRU would be between 1700-3000. One or a few antibodies could only potentially mask a one or a few of the VASs available on an influenza particle (Figures 7.1.1; 7.1.2). Therefore it would be expected that binding of one, or a few, molecules of antibody should not negate the capacity of a virus particle to attach, if the effect of antibody is simply to create a steric barrier. The capsid of a picornavirus, polio, consists of 60 protomers (Rueckert, 1976) each of which is composed of four proteins, VP1-4. It is proposed that picornaviruses (Rossmann et al., 1985; Hogle et al., 1985) have a conformational pocket on each protomer which functions as a VAS. Icenogle et al. (1983) showed that the maximum number of monoclonal IgG molecules that would bind to a poliovirus particle was 30, but that significant neutralization occurred when 3 IgG molecules were bound. It was suggested that each IgG molecule in this situation bound bivalently to 2 epitopes on 2 different protomers related by a two-fold axis. Thus when 3 antibody molecules bound there were 54 other VAS on 54 other protomers available for cell-virus interaction. Since it has been shown that poliovirus-antibody complexes can attach to cells (Mandel, 1967) it is reasonable to consider neutralization is brought about by the failure of the virus to function normally at the penetration or uncoating stage (section 9.2.1; section 9.2.2).

The determination of the number of molecules needed for neutralization is therefore an important consideration and evidence that only one or a few IgG molecules are able to block only one or a few potential binding sites questions the classical paradigm that the function of antibody is to block viral attachment (Lachmann, 1985).

The portion of infectivity that remains after reaction between a population of virus and neutralizing antibody has been termed as the non-neutralized or persistent fraction (Dulbecco et al., 1956) (Figure 8.1). The emergence of a persistent fraction can be due to one of a combination of factors (Reviews: Daniels, 1975; Mandel, 1979). These factors may involve the avidity of the antibody (Lafferty, 1963a,b). He found that the neutralization mediated by a low avidity antibody was undone by dilution of the antibody-virus complex. Dulbecco et al. (1956) postulated that certain non-neutralizing antibodies in a polyclonal population of IgG may bind to the virus and prevent the attachment of neutralizing antibody. Mandel (1958) found that anti-IgG neutralized the persistent fraction of poliovirus, indicating that non-neutralizing antibody was bound to the virus. Steric factors between virus bound antibody and free antibody could determine the persistent fraction. Ashe and Notkins (1967) showed that HSV-IgG complexes resistant to further neutralization by intact IgG could be neutralized by Fab' fragments. It was presumed that these smaller molecules can gain access to the

virus particle unavailable to the whole IgG molecule. Non-neutralizing antibody in binding to non-critical sites may exclude the binding of neutralizing antibody to critical sites (Daniels, 1975). Massey and Schochetman (1981) used [ $^{125}\text{I}$ ]-labelled monoclonal IgG to Kirsten sarcoma virus to show that certain monoclonal IgGs while binding to the virus did not neutralize. Furthermore they prevented the subsequent attachment of neutralizing monoclonal IgGs as judged by [ $^{125}\text{I}$ ]-labelled monoclonal IgG binding in neutralization assays. Aggregation caused by immunoglobulin allowing virus in the centre of the aggregates to escape neutralization was reported with echovirus type 4 and cited as a possible cause of the persistent fraction (Wallis and Melnick, 1967). However, filtration to remove aggregated virus does not prevent the formation of persistent fractions with VEEV (Hahan, 1970), with poliovirus (Lewenton-Kriss and Mandel, 1972) as well as echovirus type 4 (Kjellen and von Zeipel, 1984).

It should be noted that all studies with polyclonal antibody are difficult to interpret because of heterogeneity of the antibody population (i.e. specificity, affinity, non-neutralizing antibody etc.). The emergence of escape mutants in the incubation of virus populations with monoclonal antibodies indicates that a proportion of the persistent fraction consists of variants that escape neutralization on account of the absence of specific antibody to neutralization epitopes peculiar to the variants.

Studies with VSV (Kjellén and Schlesinger, 1959) and with echovirus type 4 (Kjellén and von Zeipel, 1984) showed that the non-neutralizable fraction depends not only on the virus-antibody reaction but also on the cells being inoculated (section 9.1).

The biological implications of the persistent fraction are manifest in the occurrence of infectious virus-antibody complexes in the blood of pigs infected or immunized with African swine fever virus (ASFV) (De Boer, 1967; Hess, 1981). Infection or immunization with ASFV produces non-neutralizing antibodies and hypergammaglobulinaemia in pigs but does not induce neutralizing antibodies (Pan et al., 1970). Specific antibody produced in the infection of mink with Aleutian disease virus (ADV) also fails to neutralize infectivity (Review: Porter and Cho, 1980). ADV is also characterized by circulating immune complexes and hypergammaglobulinaemia. Caprine arthritis-encephalitis virus (CAEV) fails to induce neutralizing antibody in its natural host (goat) or in sheep (Narayan et al., 1984). The biological reasons for these observations have not been fully explained but the apparent failure to neutralize virus may be due to the lack of appropriate antigenic epitopes (either qualitatively or quantitatively) or a constitutive deficiency or impairment of the host immune defence. Certain non-neutralizing antibodies as well as impeding the access of neutralizing antibody can form damaging circulating immune complexes. This can occur notably in CAEV and ADV infections, and lymphocytic choriomeningitis virus (LCMV)-antibody complexes in mice persistently infected with LCMV



leads to manifestations of immunologic injury (Oldstone and Dixon, 1967; Oldstone et al., 1972).

#### 9.1. Influence of host cell on neutralization of virus

In experiments with VSV, Kjellén and Schlesinger (1959) showed that host cells may influence the extent of neutralization. VSV produced similar titres on CEF and leukaemic bone marrow cells before neutralization but after incubation with antibody there was up to a thousand fold higher titre on CEF cells compared to the leukaemic bone marrow cells. This phenomenon has also been observed with echovirus type 4 (Kjellén and von Zeipel, 1984). The extent of measurable neutralization of echovirus by polyclonal serum was dependent upon whether the host cell used to assay residual infectivity was rhabdomyosarcoma (RD) cells or green monkey kidney (GMK) cells.

Recent studies on the neutralization of enterovirus 71 by polyclonal IgG (Kjellén, 1985) showed that the extent of neutralization by different populations of IgG varied from one host system to another. Populations of IgG were separated by difference in absorption to protein A. One population of antibody reduced infectivity by interaction at exposed antigenic sites (residual infectivity could be measured after virus and antibody had been allowed to interact). A second antibody population only attached to antigenic sites after interactions between

enterovirus 71 and host cells. This second type of antibody activity could be detected on RD cells but not on GMK cells. The inference from these studies was that in order to neutralize, the second type of antibody required a particular host system. Critical sites on the virus only become vulnerable to antibody during virus-host interactions, probably via an allosteric modification of the VAP.

In assessing the neutralization of La Crosse (LAC) virus by a panel of monoclonal IgGs specific for the G1 glycoprotein it was found that antibodies showed host-dependent neutralization ability (Grady and Kinch, 1985). One antibody was neutralizing only when virus was assayed on BHK-21 cells. Another antibody was neutralizing only when cells from *Aedes albopictus* (a species of mosquito that is a vector of LAC virus) were used. Four other monoclonal antibodies showed no host dependency. The monoclonal antibody that neutralized only when mosquito cells were used recognized an epitope in a region of the G1 glycoprotein that was distinct from those recognized by the other antibodies, highlighting that this region may be an important determinant in the interaction of virus with its arthropod vector. It may be relevant that virus in mosquitos only comes into contact with antibody when it is ingested in a vertebrate blood meal.

These experiments highlight the need for studies considering the mechanism of protection by antibody of differentiated target cells involved in infection of a virus with its natural host. For

example, human adenoid organ culture can provide an attractive model to study both the pathogenesis of influenza A infections in man and the resultant mechanisms of neutralization in the host (Edwards et al., 1986). Buchmeier et al. (1984) found that one neutralizing monoclonal antibody to murine hepatitis virus type 4 (quantified by plaque reduction on L-24 cell monolayers) did not protect mice against challenge whereas another of the same sub-class, but directed against a different epitope, did protect. This illustrates that mechanisms observed in cultured cells may indeed differ from those in the actual host.

## 9.2. Mechanisms of the virus neutralization reaction.

Although the neutralization of viruses has been studied empirically for many years it is not well understood at the molecular level. Virus neutralization is a tripartite system involving the virus the target cell and the antibody. It is becoming increasingly apparent that a given mechanism of neutralization has to be strictly defined as to the nature and origin of the host cells, the virus, the class and clonal nature of the neutralizing antibody, and the conditions of the assay (Review: Dimmock, 1984). However, the more defined and controlled an experiment becomes (i.e. with the use of monoclonal antibodies and monoclonal cells) the greater the divergence from the events that occur in infection of the natural host.

### 9.2.1. Ability to prevent the first step in virus infection: Attachment.

A review of the observations on the effect of antibody on the attachment of an animal virus to its host cell indicates that while in certain cases antibody can inhibit attachment to the host cell, there are many instances where neutralized virus does attach to cells.

Rubin and Franklin (1957) studying the neutralization of Newcastle disease virus (NDV) found that the capacity of NDV to

attach to cells decreased with increasing antibody concentration. Studies by Smith et al. (1961) with vaccinia virus and Morgan et al. (1968) with HSV showed that neutralized viruses of both types were less efficient in attaching to cells than non-neutralized controls. Other studies have demonstrated that neutralized virus can attach to cells. For example, rabbit pox virus neutralized by polyclonal sera attached to HeLa cells (Joklik, 1964), influenza virus neutralized by polyclonal sera bound to and penetrated endodermal cells of the chorioallantoic membrane in chicken eggs (Morgan and Rose, 1968) and influenza virus neutralized by polyclonal and monoclonal IgG attached normally to CEF cell monolayers (Possee, 1981; Possee and Dimmock, 1981).

The use of monoclonal antibodies combined with increased understanding of virus structure and function has allowed investigation of the mechanisms of neutralization. For example, studies by Lee et al. (1981) determined that the sigma 1 protein of reovirus type 3 is the VAP for L cells and that neutralizing monoclonal antibody to sigma 1 prevented attachment. However, there was no quantitation between the efficiency of neutralization and inhibition of attachment. NDV can be neutralized by monoclonal antibody to the HN protein or its F protein (Russell et al., 1983). Monoclonal antibody to the F protein neutralized virus at a stage between attachment of virus and its entry into MDCK cells (Russell, 1986). Neutralization of NDV (Ulster) via two epitopes on HN (HN-1 and HN-2) was enhanced when the virus was assayed on sialidase-treated

Madin-Darby bovine kidney (MDBK) cells which had been partially denuded of receptor function (Russell, 1986). This implied that monoclonal antibodies to both HN epitopes were competing with cell receptors for virus and had more chance of neutralizing when virus took longer to make the necessary attachments which preceded fusion. Neutralization via monoclonal antibody to F was not enhanced by treating cells with sialidase.

The envelope of the bunyavirus, La Crosse (LAC) virus contains two glycoproteins: G1 and G2 (Gentsch et al., 1980), however infectivity is dependent on G1 and only anti-G1 antibody neutralizes infectivity (Kingsford and Hill, 1983). The topography of the antigenic sites of G1 was determined using a panel of monoclonal antibodies and it was revealed that there were 7 regions (A through G) on G1 involved in neutralization (Kingsford and Hill, 1983; Kingsford and Ishizawa, 1984). IgM to G1 blocked binding of monoclonal IgGs to 4 of the regions on G1 suggesting that the glycoprotein is folded back on itself. A maximum of 40-45% of infectious virus inoculum bound to BHK-21 cells by 2h at 1°C. Polyclonal IgG to LAC reduced attachment by 50%. When virus was pre-incubated with a neutralizing monoclonal IgG to one of each of the 7 antigenic regions on the G1 only antibodies to region A reduced attachment (by 92%). Although monoclonal IgGs to regions F and G neutralized greater than 90% of the virus they only inhibited attachment by 6 and 12% respectively. IgGs to regions B, C, and E showed intermediate degrees of neutralization and inhibition of binding. The results

indicate that antibodies binding to regions A and B probably neutralize LAC virus by sterically preventing attachment of virus to CRUs. Antibodies to sites distal from these regions (specifically to F and G) have little effect on attachment and therefore must inhibit a different step(s) in LAC virus replication as their mechanism of action.

Fuller and Spear (1985) tested the ability of polyclonal and monoclonal IgG directed to individual HSV type 1 (HSV-1) glycoproteins to inhibit the attachment of radiolabelled HSV-1 to human epidermoid carcinoma (HEp-2) cell monolayers. Some polyclonal IgGs specific for glycoprotein (g)D or gC and monoclonal IgGs for gD and gC inhibited HSV-1 attachment. However, the monoclonal IgGs to gD that were most effective at inhibiting HSV-1 attachment had only weak neutralization activity. The most potent anti-gD neutralizing antibodies had very little or no effect on the attachment of HSV-1 at concentrations significantly higher than those required for neutralization. These results suggest that although some anti-gD antibodies can neutralize by blocking attachment, a more important mechanism of neutralization by anti-gD antibodies may be interference with a step subsequent to attachment, possibly penetration. The neutralization activity of the anti-gD antibodies correlated strongly with their ability to inhibit Vero cell-cell fusion induced by HSV-1 (Noble et al., 1983). It is speculated that these antibodies block the spread of HSV-1 infection by inhibiting virus induced cell fusion. Anti-gB did

not inhibit cell fusion.

Poliovirus neutralized by polyclonal IgG was found by Mandel (1967) to bind to HeLa cells. At low concentrations of antibody the poliovirus-antibody complexes attached to cells as well as if not better than that of non-neutralized virus. Increase in attachment was proportion to the time that the mixtures were incubated with cells. The time dependent increase in attachment was seen with IgG but not with IgM. When higher concentrations of antibody were used attachment decreased but was never completely inhibited. In a more recent study Emini et al. (1983a) found that only one out of 12 monoclonal IgGs inhibited the attachment of radiolabelled poliovirus to HeLa cells under non-saturating but neutralizing conditions. Also poliovirus neutralized by monoclonal antibody that did attach also penetrated the cells. Emini et al. (1983a) found that polyclonal IgG failed to prevent the attachment of poliovirus but inhibited penetration. It is proposed that the VAS of picornaviruses is located in a pocket 2.5 nm deep on the surface of the protomer (Rossmann et al., 1985; Hogle et al., 1985). This region is not immunogenic on the intact virus and an antibody molecule with Fab arms 3.5nm in diameter would have difficulty in gaining access to this region, as its entrance would be blocked by the residues on the rim of the pocket. This topography could permit the virus to retain its ability to seek out its specific CRU despite neutralizing antibody being bound to other regions on the capsid; thus reconciling the observations of neutralizing virus binding. In



studying the neutralization of another picornavirus, human rhinovirus-14 (HRh-14), it was found that only one out of eight monoclonal antibodies inhibited the attachment of [ $^{35}\text{S}$ ]-labelled virus (R.J. Colonno, unpublished data). However the degree of neutralization of HRh-14 has been found to be dependent on the antigenic region to which the antibody associates (Sherry and Rueckert, 1985) suggesting the possible utilization of different mechanisms for neutralization.

Transmissible gastroenteritis virus (TGEV) neutralized to more than 99.99% by either polyclonal sIgA or IgG attached to swine testis cells or pig kidney cells (Nguyen et al., 1986). Neutralizing sIgA also increased the attachment of virus compared to that of non-neutralized virus.

Schlegel and Wade (1983) drew attention to another possible outcome of the interaction of neutralized virus to cells. Neutralized VSV attached to host cells via novel (lipid) receptors not used by non-neutralized VSV. The rate of attachment of neutralized VSV was equivalent to or greater than that observed for infectious VSV.

The binding of neutralized influenza virus to erythrocytes was inhibited by neutralizing IgG although the same neutralized virus bound normally to CEF cells (Possee, 1981; Possee and Dimmock, 1981).

Haemagglutination inhibition (HI) forms a useful index of immunity to infection (Hobson et al., 1972), however, HI and neutralization are not exclusive. Haemagglutination of influenza virus A/duck/Hokkaido/5/77 (H3N2) was not inhibited by neutralization positive sera of mice and ducks (Yoden et al., 1982). Kida et al. (1982) demonstrated that efficient neutralization of influenza A/seal/Massachusetts/1/80 (H7N7) with concomitant lack of HI activity. These results could indicate that the CRUs on susceptible cells and erythrocytes differ in topography and interaction with virus and that erythrocytes may be a poor model for infection (section 3.1). Glycophorin A molecules (which carry CRUs for influenza virus; section 3.1) on the erythrocyte surface lie amongst a web of carbohydrate. Carbohydrate structures reach to a distance of 15nm from the bilayer membrane and allowing for the possibility of free random coiling of the peptide portions of the membrane glycoproteins this layer may perhaps reach a thickness of 50nm (Viitala and Jarnefelt, 1985). The carbohydrate cover therefore may constitute an obstacle to close contact, a situation that may not be reflected on the surface of susceptible cells allowing neutralized virus to attach (Possee, 1981, Possee and Dimmock, 1981). It was concluded that neutralization of infectivity in such cells was due to the impairment of a post attachment event. There are twelve regions (antigenic sites) on the influenza H3 HA trimer to which neutralizing antibody may bind (section 2.2). The VAS is located at in a pocket (section 2.2) at the tip of each monomer (i.e. three VASs per HA spike). This region is not

immunogenic on the native influenza virus particle. In the same manner described for the VAS of picornaviruses this region is inaccessible to the Fab' arms of IgG and possibly when neutralizing IgG is bound to the HA is inaccessible to the CRUs on erythrocytes, but not the CRUs on susceptible cells.

There are discrete functional domains on the reovirus sigma 1 protein (Spriggs et al., 1983) and it has been found that some neutralizing monoclonal IgGs neutralize infectivity but do not inhibit viral haemagglutination both properties of sigma 1. Monoclonal antibodies with the reciprocal properties were also found. Anti-G2 antibody can inhibit the haemagglutination of LAC virus despite the fact that G1 is the attachment protein and anti-G2 does not neutralize (Kingsford and Hall, 1983). The effect is probably due to a steric blocking of the binding sites for erythrocyte CRUs on the G1. A similar situation has been described for influenza virus by Webster et al., (1982) who proposed that the HI activity associated with an antibody to NA was due to the steric blocking of sites on the HA. Anti-NA did not neutralize infectivity.

The attachment of neutralized virus to cells should not be confused with the well documented phenomenon of antibody dependent enhancement (ADE) of infection in certain cells (Hawkes, 1964). This can be due to an interaction between virus particles coated with sub-neutralizing concentrations of antibody and homologous Fc receptors on the surface of susceptible cells

(Halstead and O'Rourke, 1977; Halstead, 1980). A combination of antiviral antibody, complement, and receptors for components of the complement system on cells can also lead to ADE (Cardosa et al., 1983).

ADE has been extensively studied in flaviviruses. The ADE of dengue and West Nile viruses is due to the presence of sub-neutralizing concentrations of IgG in association with cells of the monocyte/macrophage lineage (Halstead and O'Rourke, 1977) which are rich in Fc receptors. ADE has been reported with other viruses apart from flaviviruses, including some bunyaviruses, alphaviruses, reoviruses and coronaviruses (Review: Porterfield and Cardosa, 1984). King et al. (1984) demonstrated ADE of rabies virus infection in mouse macrophages and the antibody mediated rabies "early death" phenomenon in mammals (Prabhaker and Nathanson, 1981) may be a manifestation of ADE.

It was reported that enhancement could be produced in BHK-21 cells infected with the alphavirus, Getah virus (Kimura et al., 1981). However these observations were not confirmed in a number of experiments comparing interactions of viruses with macrophages and BHK-21 cells (Porterfield and Cardosa, 1984). There is no evidence that macrophages contribute to the attachment of neutralized influenza virus to CEF cell monolayers (Dimmock et al., 1984).

### 9.2.2. Neutralization mechanisms after attachment.

If attachment is not inhibited by neutralizing antibody loss of infectivity must be due to the inhibition of a subsequent stage of infection.

The uncoating of enveloped virus in endosomes involves destabilization via conformational changes in the external proteins sufficiently to release the virus genome into the cytosol (Review: Marsh, 1984). Such an endosomal step is involved in the entry pathway of West Nile virus (WNV) (an enveloped flavivirus) into macrophages (Gollins and Porterfield, 1935). Neutralized WNV is taken up into vesicles but neutralizing antibody inhibits endosomal fusion. (Gollins and Porterfield, 1986).

It is becoming apparent that neutralizing antibodies can interact with poliovirus in many different ways (Mandel, 1967, 1976; Icenogle et al., 1983; Brioen et al., 1983; Thomas et al., 1985; Brioen et al., 1985). Investigating further his observations that neutralizing polyclonal IgG failed to inhibit attachment, Mandel (1976) found that neutralizing antibody induced a conformational change in the poliovirus capsid protein. IgG, IgM, and Fab' fragments from IgG induced a conformational transition from state A with an isoelectric point (pI) of approximately 7 to state B with a pI of approximately 4.5.

Poliovirus is stabilized in state B by antibody and was not infectious. This transition could be mediated by a single neutralizing antibody. The change in conformation associated with the shift in pI still allowed the virus to attach to cells and penetrate but the neutralized virus was resistant to uncoating. Another manifestation of this alteration may be the enhanced affinity for binding to host cell receptor (Mandel, 1967). Substantial conformational changes also occur in the neutralization of another picornavirus, bovine enterovirus (Carthew, 1976) resulting in the exposure of VP4.

Recent studies with monoclonal antibodies (Emini et al., 1983a; Icenogle et al., 1983; Brioen et al., 1985) have illustrated that not all neutralizing antibodies induce conformational changes in poliovirus. Icenogle et al. (1983) described a monoclonal antibody (F7.12) which gave first order neutralization kinetics but required an average of 4 bound [ $^{125}$ I]-labelled F7.12 IgG molecules per virus particle to neutralize the virus efficiently. However this could be reconciled by a "one of four sites critical" model of neutralization as well as a step-wise model (where infectivity is reduced as each antibody binds). It was determined that bivalent binding of monoclonal antibody F7.12 molecules was necessary. Papain digestion of F7.12 IgG bound to virus produced Fab fragments and restored infectivity, but not through dissociation of binding as addition of anti-Fab' IgG caused re-neutralization. It was suggested that the infectivity of

poliovirus was reduced by a cross-linking of virus protomers which inhibited the dissociation of pentamers (Rueckert, 1976) that occurs in uncoating. However Brioen et al. (1983) and Thomas et al. (1985) have found that the neutralization of poliovirus brought about by a monoclonal antibody IgG (35-1f4) was due to virus polymerization (aggregation). Monoclonal antibody 35-1f4 in contrast to monoclonal antibody F7.12 was incapable of stable bivalent binding to a single virus particle. [<sup>3</sup>H]-labelled monoclonal antibody 35-1f4 was never found in association with monomeric virus particles. Virus aggregates released by papain were fully infectious. Monoclonal antibody 35-f14 failed to induce any pI shift. Emini et al. (1983a) demonstrated that neutralization-associated pI shift and neutralization via Fab' fragments were dependent on which capsid protein antibody/Fab' was attached to. The neutralization and shift in pI accompanied by the binding of IgG to VP1 was reversed by papain digestion. However, neutralization by anti-VP3 IgG (which did not cause a change in pI) was not reversed by papain digestion.

Six out of seven monoclonal antibodies to poliovirus type 1 did not prevent attachment or penetration but all prevented transcription (Emini et al., 1983a). Neutralization mediated by the monoclonal antibodies was associated with a change in the pI of the virus, however bivalent attachment to the virus was essential for neutralization by the monoclonal antibodies. Therefore these observations support the mechanism of poliovirus neutralization proposed by Mandel (1976) that neutralization

causes conformational changes in the capsid which prevents uncoating.

In assays performed at low ionic strength another mechanism of neutralization of poliovirus by monoclonal antibody 35-1f4 was observed (Brice et al., 1985). At physiological ionic strength monoclonal antibody 35-1f4 neutralized poliovirus by antibody-mediated polymerization (see above) but at low ionic strength neutralization was accompanied by disruption of the virus particles to non-infectious empty capsids devoid of antibody. The empty capsids resembled those formed by thermal denaturation of native poliovirus in their sedimentation coefficients, antigenicity (resultant particles no longer expressed the epitope recognized by monoclonal antibody 35-1f4) and pI (6.3). Therefore at low ionic strength monoclonal antibody 35-1f4 follows a "hit and run" mechanism of neutralization.

Possee et al. (1982) demonstrated that virion RNA from neutralized influenza virus accumulated in the nuclei of CEF cells at the same rate as that of non-neutralized virus. The similarity in the kinetics of migration of the RNA from the cytoplasm fraction of the cell to the nuclear compartment suggested that neutralized and non-neutralized influenza virus follow the same route. Transcription of viral RNA was not detected in cells inoculated with neutralized influenza virus and *in vitro* studies with monoclonal antibodies showed that the activity of the viral transcriptase was impaired



by neutralization. It was suggested that the binding of a neutralizing antibody to the HA initiates a signal possibly via a conformational change which is transmitted across the envelope via the transmembrane segment of the HA2 to the transcriptase. However, the drop in transcriptase activity (three to tenfold) was not in proportion to the 99.99% neutralization achieved. Similarly neutralizing antibody had little effect on messenger RNA primed transcription (R. Rigg and N.J. Dimmock, unpublished data). However inhibition of transcription *in vitro* was dependent on intact virus particles, as addition of antibody after virus had been treated with detergent did not result in inhibition of transcription (A.S. Carver and N.J. Dimmock, unpublished data).

The situation that neutralizing antibody does not inhibit the attachment, penetration or transport of the influenza virus RNA to the nucleus does not preclude the possibility that an infectious process such as uncoating is defective. Recent evidence suggests that there is a second uncoating step (Bukrinskaya et al., 1982) in which M is removed from the viral core. This could be inhibited by neutralizing antibody resulting in delivery of a core which is unable to release its genome in a state suitable for transcription (R. Rigg and N.J. Dimmock, unpublished data).

Conformational changes mediated by the binding of an antibody to a virus have been implicated notably in the

neutralization of bovine enterovirus (Carthew, 1976) and poliovirus (Mandel, 1976; Emini et al., 1983a,b). However, the involvement of antibody-mediated conformational changes in the neutralization of influenza virus involves the further concept that antibody binding to an external protein (the HA) affects the processes dependent on internal viral proteins.

The ability of antibody to mediate conformational changes in antigens is well documented. Antibody has been observed to cause structural alterations in enzymes which in turn can effect enzyme activity (Rotman and Celada, 1968; Zimmerman et al., 1971; Erickson, 1974). Proteins are in a perpetual state of motion, so that conformation of a particular molecule cannot be specified absolutely except as an average of a multitude of available conformations, and it is convenient to visualize them as minature mechanical devices (Review: Cooper, 1980). In simplistic terms when the size of an IgG or IgM molecule is considered it is not difficult to envisage a conformational change in the antigen to which it binds. An IgG molecule has dimensions not much less than that of the HA of influenza virus (Figures 7.1.1; 7.1.2). Competitive binding radioimmunoassays on isolated influenza virus HA showed that the binding of certain monoclonal antibodies increased the binding of other monoclonal antibodies to different epitopes due to conformational changes within the HA (Lubeck and Gerhard, 1982). An enhancement effect of some monoclonal antibodies on the binding of other monoclonal antibodies was observed in studies on the interaction of antibody with the major

surface glycoprotein of VSV (Lefrancois and Lyles, 1982). The reason for the increased attachment of antibody was probably the result of allosteric modifications of the glycoprotein induced by the binding of the first monoclonal antibody.

In considering a mechanism of neutralization of influenza, an aberration of secondary uncoating or an effect on transcription could conceivably occur only if a conformational change due to the binding of neutralizing antibody is transmitted into the viral core. Many properties of proteins, including regulation of their activity depend on transmission of conformational changes between distant sites (Review: Cooper, 1980). However little is known about the detailed mechanisms of these processes. Studies of the conformational changes in insulin molecules (Chothia et al., 1983) demonstrate that because of the packing of alpha helices the extent to which a conformational perturbation can be accommodated locally is limited (to shifts of no more than 0.15nm). This limit to the dissipation of helix movements propagates the transmission of conformations over long distances through the molecule. The transmission of conformational changes along in the insulin molecule can even be amplified. Although individual proteins have individual mechanisms of function these ideas of transmission due to helical backbone rigidity should generally apply to conformational changes (including allosteric transitions) in proteins and such a process may occur on the binding of neutralizing IgG to influenza HA.

### 9.2.3. Cooperative neutralization

Studies with neutralizing monoclonal antibodies have highlighted the phenomenon of cooperative neutralization where the binding of two monoclonal antibodies results in a greater loss of infectivity than the combined individual effects. Enhanced neutralization of LAC virus (Kingsford and Ishizawa, 1984) resulted from the cooperative binding of two monoclonal antibodies to different epitopes on G1. However unlike the synergistic binding of monoclonal antibodies observed with monoclonal antibodies to influenza HA by Lubeck and Gerhard (1982) there was no increased binding of one antibody due to the presence of the other. It was suggested that the increased neutralization resulted from conformational changes in the virus protein as these monoclonal antibodies did not significantly reduce attachment. Russell (1986) demonstrated that the binding of monoclonal antibodies to different epitopes, HN-1 and HN-2 on the HN of NDV (Ulster) resulted in cooperative neutralization. These results paralleled those observed with LAC virus (Kingsford and Ishizawa, 1984) in that neither monoclonal antibody to the HN enhanced the binding of the other. After finding that sialidase-treatment of cells increases the neutralization of NDV (Ulster) the method of neutralization was thought to be cooperative interference of viral attachment via HN-1 or HN-2. However it is not known whether the interference resulted from steric hindrance of attachment or induction of a conformational

change in the HN.

### 9.3. Conclusion.

In view of the different mechanisms of neutralization observed with monoclonal antibodies to HSV, LAC virus and poliovirus it is inferred that the mechanism of neutralization in the natural animal host will depend on the affinity, abundance of the class of immunoglobulin and relative abundance of antibody within a class to any particular antigenic site. Neutralization by polyclonal sera is likely to be a sum of a number of different types of neutralizing mechanisms.

The different mechanisms of viral neutralization are dependent on the interactions between virus, antibody and cell. It is becoming apparent that neutralizing antibody may affect any of the early events in virus infection. The lack of a general mechanism for the neutralization of viruses by antibody may be paralleled by the inhibition of infection of other pathogens by antibody. For example, different IgGs to the circumsporozoite protein of the protozoan *Plasmodium falciparum* (the malarial parasite) exert their protective effect on the sporozoite stage of the parasite at three points: attachment to liver cells, entry into the cells, and when penetration was not inhibited some antibodies effected the subsequent intracellular development of the parasites (Mazier et al., 1986).

## MATERIALS AND METHODS

### 10.1. Viruses

The avian strain of influenza virus A/fowl plague/Rostock/34 (H7N1) (FPV/R) was grown in the allantoic cavity of 11 day old embryonated hen eggs and incubated for 22h at 37°C. Allantoic fluid was collected after chilling the eggs at 4°C overnight, clarified by low speed centrifugation and snap frozen in dry ice/ethanol before storage at -70°C. This was used as inoculum. The avian influenza virus A/fowl plague/Dutch/27 (H7N7) (FPV/D) and the reassortant influenza virus A/V31/83 (H7N2) (V31) were also grown using this method. V31 had been obtained by co-inoculating CEF monolayers with a mixture of FPV/R and influenza A/X49 (H3N2) using the method of Rott et al. (1976) described in section 10.15. A/X49 is reassortant human strain and as such was grown in eggs for 48h at 33°C. A/WSN (H1N1) was a gift from L. McLain (Warwick).

The infectivity of FPV/R and V31 were determined by plaque assay on primary chick embryo fibroblast (CEF) cell monolayers under 0.9% agar in 199 medium (Flow Laboratories, Irvine, U.K.), containing 5% newborn calf serum (NCS) (Gibco-BRL Ltd., Paisley, U.K.), and expressed as plaque-forming units (PFU)/ml (Dimmock and Watson, 1969). Cell monolayers were incubated at 37°C for 3 days before staining for plaques with 0.02% neutral red (Flow Laboratories) in phosphate buffered saline (PBS). Influenza virus A/WSN required the replacement of NCS with 0.1% BSA and the

addition of DEAE/dextran to 0.01% in the overlay medium.

The haemagglutination titre of influenza virus was assayed by making doubling dilutions in PBS and adding chicken erythrocytes to 0.5% (Borland and Mahy, 1968). This is equivalent to  $7.3 \times 10^6$  erythrocytes per well. The titre was determined by estimating the 50% end-point between full agglutination and zero agglutination after 30 minutes at room temperature. Titre was expressed as "haemagglutination units" (HAU).

Semliki Forest virus (SFV) was a gift from A.D.T. Barrett (Warwick). Infectivity was assayed by plaque assay on CEF cell monolayers under 0.9% agar in 199 medium with DEAE dextran at 0.04%. Cell monolayers were incubated for 2 days at 33°C.

#### 10.1.1. Purification of influenza virus grown in eggs.

The procedure used was essentially that of Kelly and Dimmock (1974). Allantoic fluid harvested from the embryonated eggs was clarified by low speed centrifugation and then the virus was pelleted at 75,000g for 90 min at 4°C. After soaking in PBS for 18h the pellets were resuspended, residual cell debris removed by low speed centrifugation and the virus suspension loaded onto a 60 ml 15-45% (w/v) linear sucrose gradient in 1 mM Tris-HCl, 150 mM sodium chloride (pH 7.4) (TBS). After centrifugation at 60,000g for 90 min at 4°C the visible virus band was harvested,



diluted in PBS and loaded onto a 30 ml 30-70% (w/v) linear sucrose gradient in TBS. Following centrifugation at 60,000g for 18h at 4°C the virus band was harvested and virus particles were pelleted at 75,000g for 90 min at 4°C before resuspension in PBS. Purified virus was kept frozen at 70°C.

Purified influenza virus B/Lee/40 was a gift from N.J. Dimmock.

#### 10.2. Cells.

Baby hamster kidney (BHK-21) (Flow Laboratories) cell monolayers were cultivated in roller bottles in Glasgow minimum essential medium (GMEM) (Gibco-BRL Ltd.) supplemented with 100 U penicillin, 100 ug streptomycin sulphate, and 0.295 mg tryptose phosphate per ml. and 5% vol/vol NCS (Gibco-BRL Ltd.). When confluent, cells were removed by trypsinization (0.05% wt/vol trypsin, 0.02% wt/vol versene in PBS) and reseeded in roller bottles or in 5 mm diameter Nunclon plastic Petri dishes (Gibco-BRL Ltd.) to give a concentration of  $6 \times 10^6$  cells per dish.

Primary CEF cells were prepared as described by Morser et al., (1973), seeded at  $9 \times 10^6$  cells per dish or at  $2 \times 10^6$  /200ml in roller bottles (in preparation for radiolabelling of FPV/R; section 10.3). CEF cells were stored under liquid nitrogen

since different preparations vary in their ability to take up virus at 4°C (Stephenson et al., 1978) and were always screened for this attribute before use in experiments. CEF suspension was pelleted by low speed centrifugation and resuspended in 199 medium /20% NCS/10% dimethyl sulphoxide at a concentration of  $8 \times 10^7$  cells/ml. Two ml aliquots in freezing vials were chilled at 20°C for 2 h then at - 70°C for 16 h before long term storage under liquid nitrogen. Cells were revived rapidly by thawing at 37°C and then incubating in 199 medium containing 10% NCS which was renewed after 2 h and 8 h.

Ls2s cells (Flow Laboratories) and Human foreskin fibroblasts (HFF-4) (Flow Laboratories) were cultivated in glass bottles containing GMEM (Gibco-BRL Ltd.) supplemented with non-essential amino acids (GMEM-NEAA) containing 10% v/v newborn calf serum. Cells were trypsinized (as described above) until the cells were detached. The Ls2s cells and HFF4 cells were seeded into plastic Petri dishes at  $1 \times 10^6$  and  $1 \times 10^7$  cells per dish respectively.

### 10.3. Radiolabelling and purification of [<sup>32</sup>P]-labelled influenza virus.

[<sup>32</sup>P]-labelled FPV/R was prepared in roller bottles containing  $2 \times 10^6$  CEF cells (Crumpton et al., 1978). Monolayers were washed with warm saline and incubated in phosphate-free 199

medium containing 5% dialyzed NCS for 24h before infecting with FPV/R at a multiplicity of 0.1 PFU/cell. After 1h the inoculum was removed, the cells washed with saline and 70 ml medium containing 10 mCi [ $^{32}$ P]-orthophosphate (Amersham International plc, Amersham, U.K.) added; incubation was continued for 20h. Tissue culture fluid was then clarified by centrifugation at 1000g for 10 min at 4°C and [ $^{32}$ P]-labelled virus was precipitated from tissue culture fluid on ice with 60% saturated  $(\text{NH}_4)_2\text{SO}_4$  and purified by sucrose velocity centrifugation (Dimmock et al., 1977) except that no unlabelled carrier was added. Firstly, after centrifugation at 20,000g for 20 min the virus pellet was resuspended in 3 ml PBS and loaded onto a 60 ml 15-45% (w/v) linear sucrose gradient in TBS containing 0.1% BSA. Following centrifugation at 90,000g for 90 min at 4°C, the gradient was fractionated into 2 ml aliquots and assayed for HAU titre and radioactivity. Where these activities formed a coincident peak the fractions were pooled. The virus contained in this pooled peak was diluted in PBS and precipitated with  $(\text{NH}_4)_2\text{SO}_4$  on ice with fetuin (Gibco-BRL Ltd.) (as a carrier), pelleted at 20,000g and dialyzed for 18h against PBS. Preparations were assayed for infectivity and HA. Virus was stored at - 70°C until use.

#### 10.3.1. Radiolabelling and purification of SFV.

SFV was radiolabelled with [ $^{32}$ P]-orthophosphate in roller bottles of BHK-21 cell monolayers. Cells were phosphate starved

by incubating in phosphate-free GMEM, 1% dialyzed NCS for 18h at 37°C before infection with SFV at a moi of 0.1. After 2h at 33°C the inoculum was removed and 50ml medium containing 10 mCi [ $^{32}$ P]-orthophosphate was added. Incubation was continued for 18h at 33°C. Tissue culture fluid was clarified by centrifugation at 1000g for 10 min at 4°C. Virus was pelleted at 75,000g for 4h. After soaking in PBS for 18h the virus suspension was loaded onto a 24 ml 15-45% (w/v) linear sucrose gradient in TBS. After centrifugation at 60,000g for 90 min at 4°C the visible virus band was loaded onto a 20-50% (w/v) linear sucrose gradient in TBS. Following centrifugation at 60,000g for 18h at 4°C the virus band was harvested and virions pelleted at 75,000g for 90 min at 4°C before resuspending in PBS.

#### 10.4. Estimation of the number of surface projections on FPV/R particles using an electron microscope.

The actual number of spikes cannot normally be counted in any electron micrograph of a negatively stained virus particle even when it is possible to resolve spikes over the whole visible surface of the particle since many of the spikes are superimposed. Assuming that influenza particles are roughly spherical (section 1.2; Figure 13.0.1) the number of spikes can be determined by measuring the mean spacing between the spikes and assuming that each spike is surrounded by six others (Tiffany and Blough, 1970b; Figures 13.0.2 to 13.0.5). This method of

calculation has the advantage that no very extensive array of spikes need be visible; i.e. the interspike spacing was measured in regions where the spikes were seen end on and not around the periphery (Tiffany and Blough, 1970b).

Mixtures of 109 nm diameter latex beads (Dow Latex, Serva Fribiochemica, Heidelberg, Federal Republic of Germany) and FPV/R particles were used in a ratio of 5:1 and negatively stained with 1% sodium silicotungstate (Agar Aids, Stanstead, U.K.), pH 6.5. The standard deviation of the diameter of the latex beads was given as  $\pm 2.7$  nm. The mean diameter of the FPV/R particles and the interspike separation was determined (section 13.1). These measurements were used to calculate the mean number of spikes per particle (section 13.1).

#### 10.5. Estimation of the proportion of haemagglutinin (HA) and neuraminidase (NA) spikes.

400.5  $\mu$ g ( $1.4 \times 10^5$  HAU) of FPV/R was analyzed by SDS-polyacrylamide gel electrophoresis (section 10.6). This amount of protein was required in order to obtain bands dense enough to produce measurable deflections on a Joyce-Loebl densitometer when the protein was stained with coomassie brilliant blue R-250 (Sigma London Chemical Co., Poole, U.K.). By scanning the bands at 620 nm and measuring the area ( $\text{mm}^2$ ) under the peaks the relative proportions of the HA and NA were estimated (Skehel and Schild, 1971). Coomassie blue-BSA (from 5

to 200  $\mu$ g protein) complexes on a SDS-polyacrylamide gel (section 10.6) gave a linear plot of the area under peak against  $\mu$ g protein and followed Beers law. Relative concentrations within a mixture of proteins can be evaluated to an accuracy better than  $\pm$  3% (Fazekas de St. Groth et al., 1963).

#### 10.6. Polyacrylamide gel electrophoresis.

Proteins from purified virions or purified immunoglobulins were analyzed by the Laemmli system of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Final acrylamide concentrations in separating and linear gradient gels are detailed where pertinent. Samples were reduced by boiling for 5 min in 2% w/v SDS (BDH Chemicals Ltd. Poole, U.K.), 5% w/v beta mercaptoethanol, 5% w/v glycerol,  $1.25 \times 10^{-3}$  % w/v bromophenol blue in 10 mM Tris/HCl (pH7.4). For investigation of multichain complexes samples were electrophoresed without reduction by substituting 10 mM iodoacetate for the beta mercaptoethanol in the solubilizer buffer. Electrophoresis was carried out until the bromophenol blue reached the bottom of the gel. Coomassie Brilliant Blue R (0.025%) in 50% (w/v) methanol, 5% (v/v) acetic acid was used to locate protein bands after electrophoresis.

#### 10.7. Isolation of immunoglobulin G (IgG) from serum or ascites.

The source of polyclonal IgG was hyperimmune rabbit serum. The samples of antiserum to the HA (H7) of FPV/R were gifts from R.D. Possee and A.S. Carver. These had been raised in rabbits by inoculation with FPV/D (H7N7) which has an HA antigen which is very closely related to that of FPV/R and an antigenically

unrelated NA.  $10^5$  HAU of purified FPV/D were injected intravenously at days 1 and 22 (boost) and serum obtained by bleeding at 5, 6, or 7 days post-boost. Sera from three rabbits designated WR6; WR26; WR45. Subsequent experiments used WR6 and WR45 sera from day 6 post-boost and WR26 from day 7 post-boost. Latterly a reassortant influenza virus (section 10.15) A/V31/83 (H7N2) (V31) was used in the same manner to produce hyperimmune sera specific for the HA of FPV/R (sera designated WR69; WR70; subsequent experiments using sera from days 6 and 7 post-boost respectively). All detectable antibodies to type specific antigens NP and M protein were removed from sera by absorption to ether disrupted A/X49 (H3N2) when required.

Antiserum specific for FPV/R neuraminidase (NA) was a gift from A.S. Carver. This had been raised in rabbits by the inoculation with influenza A/chicken/Scotland/59 (H5N1) which has a NA antigen very closely related to the NA of FPV/R but a unrelated HA. Ascitic fluid containing neutralizing murine monoclonal IgG to H3 HA (the preparation was designated 171/1) was a gift from R.G. Webster, St. Jude Childrens' Research Hospital, Memphis, TN, U.S.A. Antisera specific for N2 was a gift from A.S. Carver. This was rabbit hyperimmune sera against a recombinant influenza virus (H1N2) from a reassortment between influenza virus A/NWS (H1N1) and A/Japan/57 (H2N2) which has a NA antigen related to that of A/X49.

Ascitic fluid containing the neutralizing murine monoclonal



antibodies to FPV/R HA (designated: HC2; HC10; HC58; HC61, all IgG) were kindly provided by A.R. Douglas and J.J. Skehel (N.I.M.R., London, U.K.). Rabbit and mouse IgG were purified by affinity chromatography on a column of protein A-Sepharose CL-4B (Sigma). 1ml of antiserum or ascitic fluid was added to the column and passed through seven times with 10 mM Tris-HCl 150 mM sodium chloride (pH8) (binding buffer). The column was then washed with binding buffer until negligible protein eluted (as judged by measuring absorbance at 280 nm). Bound IgG was eluted with 0.6% sodium acetate in 150 mM sodium chloride (pH3). Fractions were promptly adjusted to pH7 with 1M sodium hydroxide. The extinction coefficient ( $E_{1\%/280}$ ) value used for calculating the concentration of mouse and rabbit IgG was 13.5 (Schultz and Heremans, 1966). The number of IgG molecules was determined using this extinction coefficient and a molecular weight (MW) for rabbit and mouse IgG of 150,000 (Kirschenbaum, 1973, Nisonoff et al., 1975) (Avogadro constant =  $6.022 \times 10^{23}$  /mol).

Purified IgG was stored at  $-70^{\circ}\text{C}$ . Pre-immune IgG (pIgG) was prepared from pre-immunization sera. Non-specific monoclonal IgG was used as control pIgG in experiments involving monoclonal IgG.

#### 10.8. Preparation of IgG fragments

Mouse monoclonal IgG purified from HC2 ascites fluid was used as a source of  $\text{F(ab}')_2$  and  $\text{Fab}'$  fragments. IgG was mixed

Figure 10.1.1.

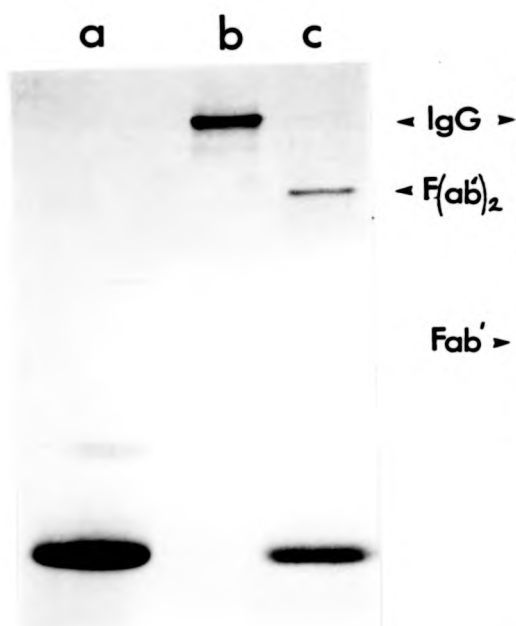


Figure 10.1.2.

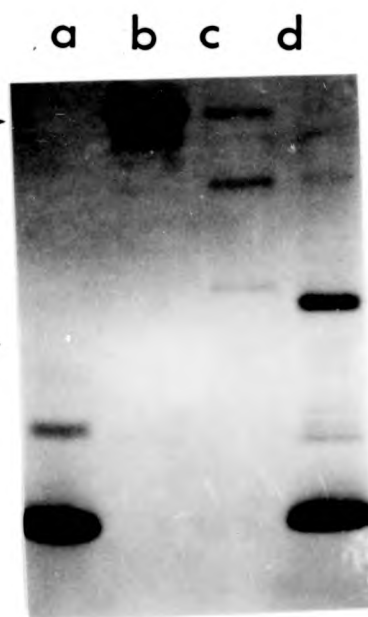


Figure 10.1.1.

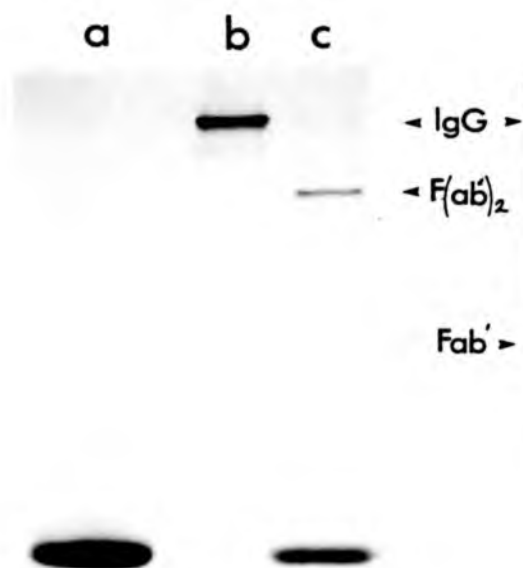
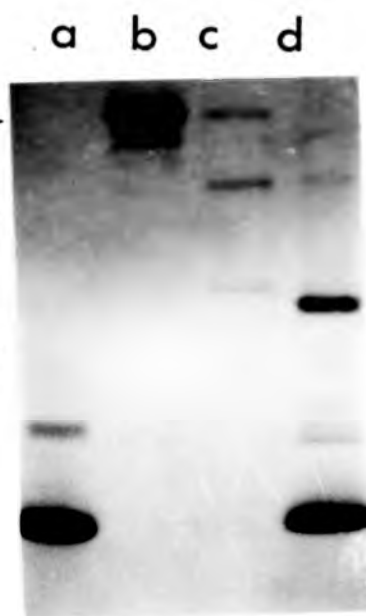


Figure 10.1.2.



**Figures 10.1.1 to 10.1.2. SDS-PAGE analysis of affinity purified IgG (section 10.7) and IgG fragments (section 10.8).** The samples were electrophoresed on the gels (10-30% polyacrylamide) in non-reducing solubilizer buffer (section 10.6). The lanes carrying IgG fragments also contained cytochrome C (12,384 MW). The gels were stained for protein with Coomassie Brilliant Blue.

**Figure 10.1.1.** Lane a cytochrome C (12,384 MW) and dimer; lane b mouse IgG (150,000 MW); lane c  $F(ab')_2$  (120,000 MW).

**Figure 10.1.2.** Lane a cytochrome C (12,384 MW) and dimer; lane b mouse IgG (150,000 MW); lane c sample before purification on protein A column after treatment with pepsin containing IgG,  $F(ab')_2$  and Fc fragments; lane d Fab' (50,000).

with pepsin (Sigma) at a ratio of 1:25 (w/w). The IgG pepsin mixture was incubated at 37°C in 70 mM acetate buffer (pH4.1) for 16h, after which time the mixture was neutralized with 2 M sodium hydroxide. The resultant mixture of F(ab')<sub>2</sub> and Fc fragments was applied to a protein A-Sepharose CL-4B (Sigma) column (section 10.7). The F(ab')<sub>2</sub> fragments did not bind to the column and were collected in the eluate. From 1.2 mg IgG in 1 ml, 0.67 mg of F(ab')<sub>2</sub> fragments was obtained. Fab' fragments were obtained from F(ab')<sub>2</sub> by partial reduction with 10 mM cysteine (Sigma) for 2h at 37 °C. Cysteine was removed by dialysis for 18h against 3 changes of PBS. F(ab')<sub>2</sub> and Fab' fragments were characterized biochemically by SDS-PAGE (Figures 10.1.1; 10.1.2 ) and stored at - 70°C with 0.1 mg/ml cytochrome C (Sigma) as a protein carrier. The strong bands seen on the gels demonstrated that F(ab')<sub>2</sub> fragments (approx. MW 120,000) and Fab' fragments (approx. MW 50,000) were obtained with high purity.

#### 10.9. Isolation of Immunoglobulin M

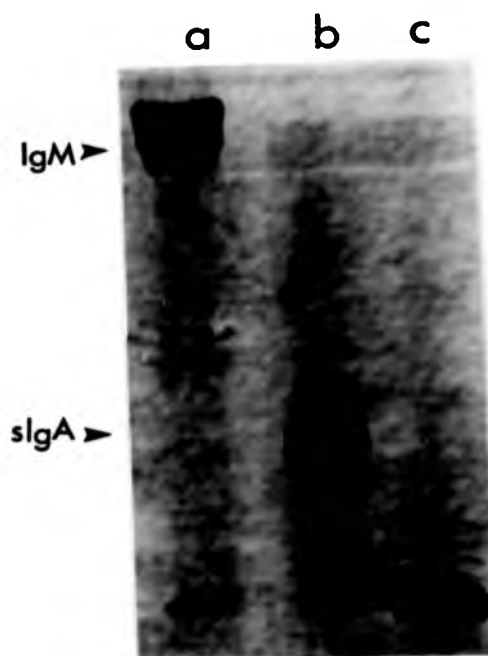
To produce IgM rabbits (2kg half-lops, Hylyne Rabbits Ltd., Northwich, U.K.) were injected with 10<sup>5</sup> HAU of purified V31 intravenously and serum was obtained daily between days 2 and 9 post-inoculation. IgM was purified either by affinity chromatography or gel filtration. Affinity chromatography was most suitable for small amounts of IgM, but gel filtration yeilded greater quantities (Table 10.1)

#### 10.9.1. Affinity chromatography purification of IgM.

An affinity column was constructed by coupling goat anti-rabbit IgM (Fc) (Nordic Immunological Laboratories Ltd., Maidenhead, U.K.) to cyanogen bromide-activated Sepharose (Sigma) (Hudson and Hay, 1980). 4ml serum was passed through the column seven times with 0.01 M Tris-HCl, 0.15 mM sodium chloride (pH8). The column was then extensively washed with the same buffer and IgM eluted with 0.6% sodium acetate in 150 mM sodium chloride (pH3). Eluted samples were dialyzed overnight against 1 mM Tris-HCl, 0.05 M sodium chloride, pH7.4, and then lyophilized. IgM was characterized biochemically by SDS-PAGE (Figure 10.2) and immunologically by immunodiffusion (Ouchterlony) against anti-rabbit IgM (Fc) antibody (Figure 10.6.1), the immunoprecipitin line between anti-IgM and the sample well confirming the identity of IgM.

IgM purified by affinity chromatography subjected to SDS-PAGE through a 5-30% w/v acrylamide gel under non-reducing conditions gave a strong band of about 900,000 MW at the expected position of the IgM pentamer (Figure 10.2). There was some contamination with smaller proteins (possibly breakdown products), but immunodiffusion (Figure 10.6.1) demonstrated that IgM was the only immunoglobulin present. Serum obtained from bleeds 5, 6 and 7 produced maximum yields of H7 specific IgM as measured by HI. Neutralizing IgM reduced the infectivity of 1.5 x

**Figure 10.2.** SDS-PAGE analysis of affinity purified IgM (section 10.9.1) (45  $\mu$ g) Lane a IgM (900,000 MW), smaller MW protein contaminants can also be seen; lane b sIgA (400,000 MW); and lane c rabbit IgG (150,000 MW); The samples were run on the gel (5-30% polyacrylamide) in non-reducing solubilizer buffer (section 10.6). The gel was stained for protein with Coomassie Brilliant Blue.



**Figure 10.2.** SDS-PAGE analysis of affinity purified IgM (section 10.9.1) (45  $\mu$ g) Lane a IgM (900,000 MW), smaller MW protein contaminants can also be seen; lane b sIgA (400,000 MW); and lane c rabbit IgG (150,000 MW); The samples were run on the gel (5-30% polyacrylamide) in non-reducing solubilizer buffer (section 10.6). The gel was stained for protein with Coomassie Brilliant Blue.

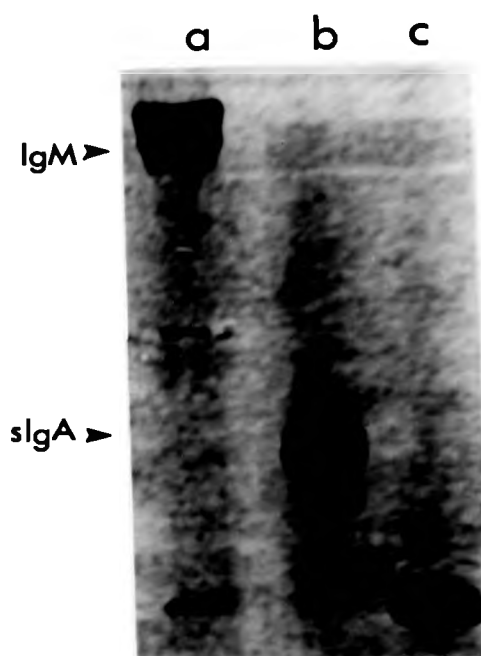




Table 10.1. Purification of immunoglobulins obtained by affinity chromatography\* (sections 10.7; 10.9.1; 10.10.1) or gel filtration @ (sections 10.9.2; 10.10.2).

\* All are from polyclonal sources unless stated.

<sup>b</sup> Monomeric IgA was obtained from 0.094 mg purified sIgA.

<sup>c</sup> Quantities are mean values.

Purified antibody <sup>a</sup>	Origin	Conc. per ml peak fraction <sup>c</sup>	Volume (ml)	Recovery/ml of original sample <sup>c</sup>
IgM *	rabbit serum	0.037 mg	1.5	$1.4 \times 10^{-2}$ mg
sIgA *	rat bile	0.153 mg	1.5	$1.15 \times 10^{-2}$ mg
monomeric IgA <sup>ab</sup>	sIgA	0.077 mg	1.0	58%
IgG *	rabbit serum	1.42 mg	1.5	1.42 mg
monoclonal IgG *	mouse ascites	1.75 mg	1.5	1.75 mg
IgM @	rabbit serum	0.165 mg	5.5	$1.14 \times 10^{-1}$ mg
sIgA @	rat bile	0.388 mg	4.5	$8.75 \times 10^{-1}$ mg

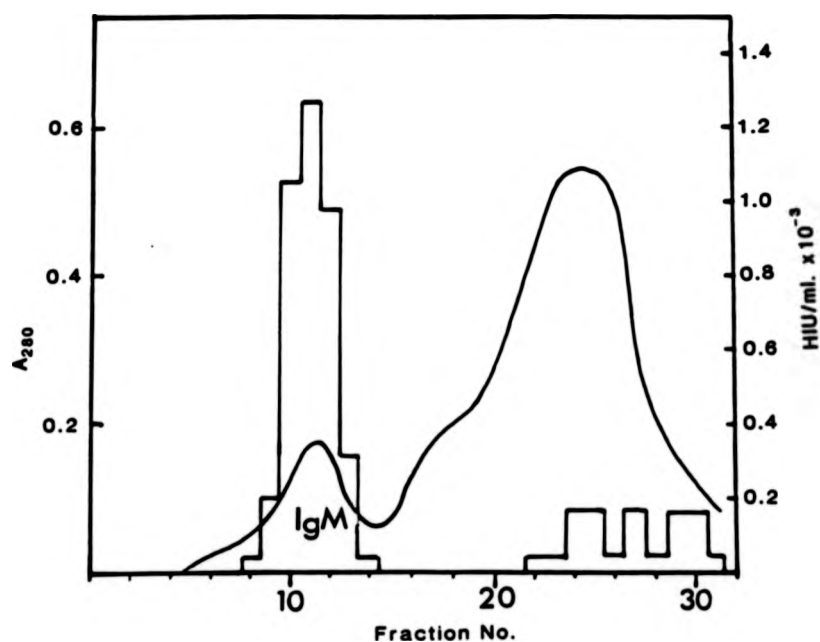
10<sup>7</sup> PFU FPV/R by 99%. IgG purified from the identical serum source had negligible specific neutralization (less than 30%).

The microimmunodiffusion technique was carried out in 1% (w/v) Noble agar in 10 mM Tris 150 mM sodium chloride (pH7.5) on 7.5 x 2.5 cm glass microscope slides. A template defined the wells into which 25 ul of 10<sup>-1</sup> dilutions of goat anti-rabbit IgM and IgG (Nordic Labs.) were placed. A 10<sup>-1</sup> dilution of sample in buffer was placed in the centre well. Antigen and antibody preparations diffused in a moist atmosphere for 3 days at room temperature. The slides were soaked for 48 h in PBS which removed soluble protein. The remaining precipitin bands were stained with 0.5% Coomassie Brilliant Blue R250 in 50% methanol containing 1% acetic acid. Excess stain was removed in the same solvent.

#### 10.9.2. Isolation of IgM by gel filtration.

IgM could also be obtained from serum by gel filtration using a Sephacryl S-300 (Sigma) column. A polyethylene glycol precipitation step was a useful preliminary procedure. 8 ml rabbit serum were dialyzed against 3 changes of 10 volumes of 2 mM phosphate buffer (pH6) at 4°C. The pH of the serum was brought to pH8 with 2 M Tris and the solution was made 6% (w/v) in polyethylene glycol 6000 (PEG) (BDH Labs. Ltd.). After 30 min at 4°C the sample was centrifuged at 2000g for 10 min at 4°C. The precipitate was resuspended using 6% PEG in 10 mM Tris-HCl

Figure 10.3. Isolation of IgM from rabbit serum on a Sephacryl S-300 column in TBS, following polyethylene glycol precipitation as determined by  $A_{280}$  (section 10.9.2). The first peak contained IgM, the shoulder of the second peak contained marker (ferritin) and smaller contaminants including IgG were eluted after this. The bar graph shows the HI titres of all fractions, as determined by the assay described in section 10.12. fractions 10, 11 and 12 were used.



(pH8.0) and recentrifuged. This procedure was repeated once again. The final precipitate was dissolved in TBS at room temperature. This in turn was centrifuged at 2000g for 10 min at 4°C and the supernatant retained. This was applied to a 30 cm<sup>3</sup> column of Sephacryl S-300 and eluted with TBS at 4°C. 2 ml fractions were collected and the absorbance monitored at 280nm. The first major peak was found to contain pentameric IgM. IgG was eluted after the peak produced by the marker protein ferritin (Sigma). (Figure 10.3). Gel filtration yielded 8 times as much IgM as affinity chromatography (Table 10.1).

IgM was stored at 4°C in 0.05% sodium azide. Bovine serum albumin (Sigma) (BSA) (to 0.5mg/ml) was added to solutions of low IgM concentration (less than 0.1 mg/ml). Pre-immune IgM (pIgM) was prepared from pre-immunization serum. An E 1%/280 of 13.5 was used to calculate the concentration of IgM (Metzger, 1970). The number of IgM molecules was determined using a MW for rabbit IgM of 900,000 (Nisonoff et al., 1975). IgM purified by gel filtration was fully classified immunologically by immunodiffusion (giving similar results to those with affinity purified IgM, Figure 10.6.1) and biochemically by SDS-PAGE (Figure 10.7).

Gel filtration produced a greater yield of uncontaminated IgM than affinity chromatography (Table 10.1). The characterization of IgM by SDS-PAGE under reducing conditions showed H<sub>mu</sub> chain (75,000 MW) and L chain only (Figure 10.7). IgM

purified by gel filtration was used for enumeration studies.

#### 10.10. Secretory Immunoglobulin A (sIgA) from bile.

$3 \times 10^5$  purified FPV/D suspended in PBS was inactivated with 0.0025% beta propiolactone (BPL) (Sigma) for 8h at 4°C, after which BPL was renewed and the mixture left for a further 24h. The virus suspensions were then dialyzed against PBS, and when tested in 11 day embryonated eggs, were found to contain no residual activity (zero haemagglutination titre). Inactivated virus was kept at 4°C until use.  $7 \times 10^5$  HAU of inactivated FPV/D was injected into the Peyer's patches (PP) of anaesthetized inbred male Wistar rats (Wistar/PC81), 10 weeks old and weighing about 200g each (Hall et al., 1979). The best strategy is to inject the largest PP carefully just underneath the membrane, being careful not to inject into the gut. In this manner approximately 15ul was injected into each PP. 7 days after inoculation the bile ducts of the rats were cannulated with a nylon catheter (0.94mm external diameter; Portland Plastics, Hythe, Kent, U.K.) and the rats were confined in Bollman restraining cages (Bollman, 1948). Bile was collected over days 7-9 postinoculation and stored at - 20°C. The inoculation procedure was carried out with the practical assistance of L. Gyure and J.G. Hall (Institute of Cancer Research, Sutton, Surrey, U.K.).

Bile salts were removed by exhaustive dialysis against 0.01M

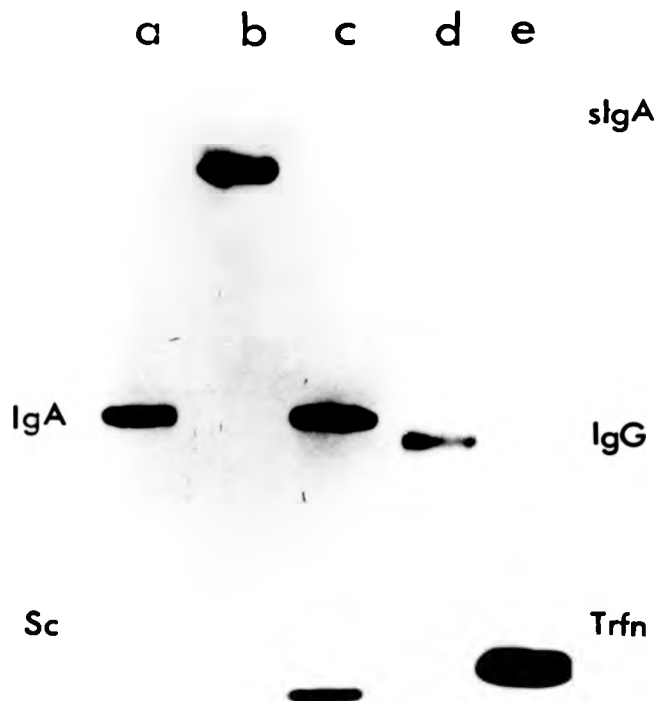
tris-HCl buffer and 0.15M sodium chloride (pH7.4), bile was concentrated 10-25 fold either on an Amicon B15 filter (Amicon Corp., Danvers MA, USA) or in centricon 10 microconcentrators (Amicon) by centrifugation at 5000g at 4°C for 70 min. IgG could be removed by passing the bile concentrate seven times down a protein A-Sepharose CL-4B column to which sIgA does not bind (Kronvall et al., 1970).

Secretory IgA was purified either using affinity chromatography or gel filtration. As with IgM purification, gel filtration provided a greater quantity of purified immunoglobulin (Table 10.1).

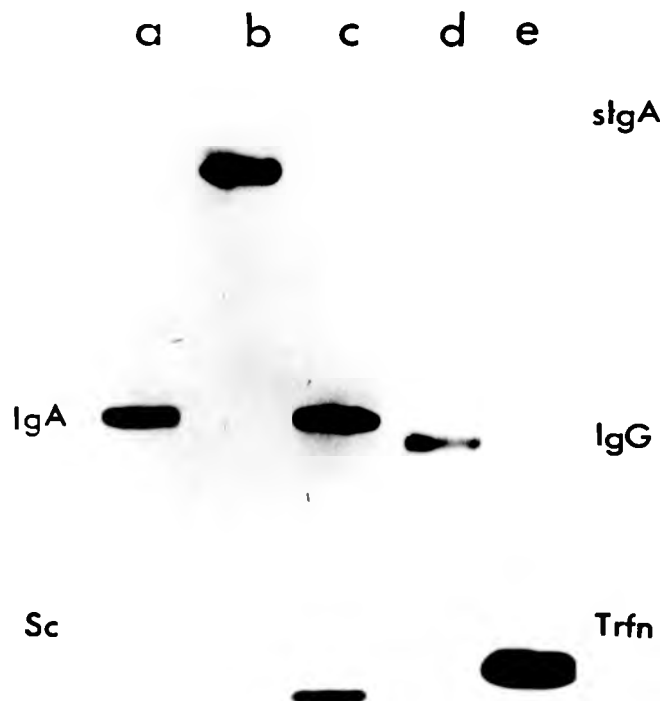
#### 10.10.1. Affinity chromatography purification of sIgA.

An affinity column was constructed by coupling goat anti-rat alpha chain antibody (Nordic) to cyanogen bromide-activated Sepharose (Sigma) (Hudson and Hay, 1980). 2ml of bile concentrate was passed through the column in 0.01 M Tris-HCl, 0.15 M sodium chloride (pH8). Secretory IgA was then eluted from the column with 0.1M ammonium hydroxide and 0.05 M diethylamine (pH11.5). Eluted samples were neutralized with HCl, dialyzed for 18h against 1 mM Tris-HCl, 0.05 M sodium chloride (pH7.4), and lyophilized. Purity of sIgA was monitored by SDS-PAGE (Figure 10.4) and immunologically by immunodiffusion (Ouchterlony) against anti alpha-chain antibody (Figure 10.6.2),

**Figure 10.4.** SDS-PAGE analysis of purified sIgA (section 10.10.1) (94 ug) before and after partial reduction with 0.01 M BME (section 10.10.3): Lane b sIgA; lane c sIgA after treatment with BME, monomeric IgA at 160,000 MW and secretory component (Sc) at 70,000 MW; lane a monomeric IgA obtained after passing the sample shown in lane c through an affinity column of anti-alpha chain IgG. MW markers are: Lane d rabbit IgG (150,000 MW); and lane e transferrin (Trfn) at 80,000 MW. The samples were run on the gel (5% acrylamide) in non-reducing solubilizer buffer (section 10.6). The gel was stained for protein with Coomassie Brilliant Blue.

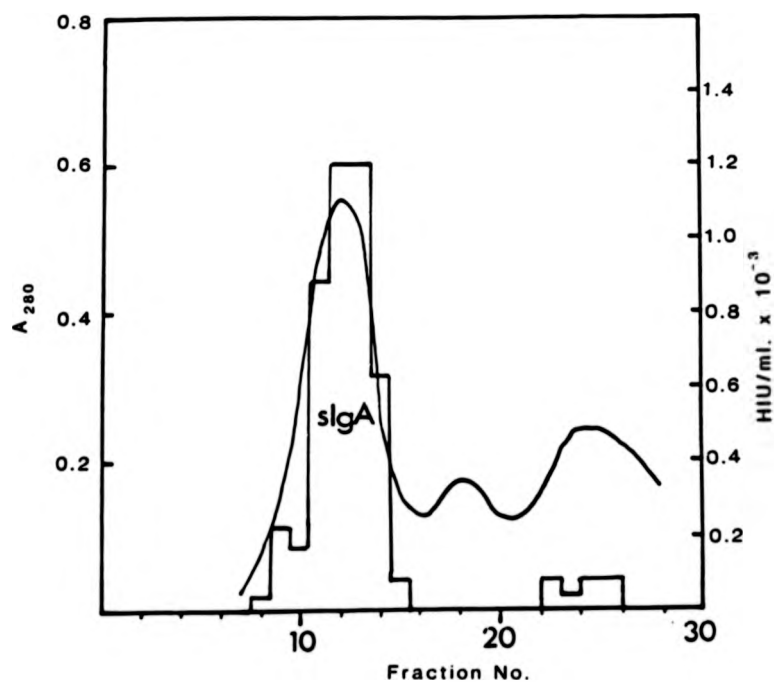


**Figure 10.4.** SDS-PAGE analysis of purified sIgA (section 10.10.1) (94 ug) before and after partial reduction with 0.01 M BME (section 10.10.3): Lane b sIgA; lane c sIgA after treatment with BME, monomeric IgA at 160,000 MW and secretory component (Sc) at 70,000 MW; lane a monomeric IgA obtained after passing the sample shown in lane c through an affinity column of anti-alpha chain IgG. MW markers are: Lane d rabbit IgG (150,000 MW); and lane e transferrin (Trfn) at 80,000 MW. The samples were run on the gel (5% acrylamide) in non-reducing solubilizer buffer (section 10.6). The gel was stained for protein with Coomassie Brilliant Blue.





**Figure 10.5.** Isolation of sIgA from rat bile on a Sephadex G-200 column in 0.1M sodium phosphate (pH 6.8) following removal of lipid and concentration of bile (section 10.10.2.). the first peak contained sIgA and the second and third peaks contained IgG and free secretory component (Sc). The bar chart shows the HI titres of all fractions as determined by the assay described in section 10.12. Fractions 11, 12 and 13 were used.



Figures 10.6.1 and 10.6.2. Immunodiffusion of affinity purified IgM (section 10.9.1) and sIgA (section 10.10.1). The microdiffusion technique was performed as described in section 10.9.1. Identical results were achieved with IgM and sIgA purified by gel filtration (section 10.9.2; 10.10.2).

Figure 10.6.1. Goat anti-rabbit IgM and anti-rabbit IgG were placed at  $10^{-1}$  dilutions in TBS in wells a and b respectively. A sample of putatively purified IgM was placed in the central well, c. A precipitin line can be seen between the centre well and well a.

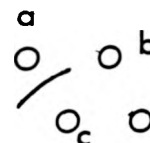
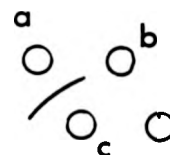


Figure 10.6.2. Goat anti-rat IgA, goat anti-rat IgG and sheep anti-rat IgM were placed at  $10^{-1}$  dilutions in TBS in wells a, b and c respectively. A sample of putatively purified sIgA was placed in the central well, d. A precipitin line can be seen between the centre well and well a.



Figures 10.6.1 and 10.6.2. Immunodiffusion of affinity purified IgM (section 10.9.1) and sIgA (section 10.10.1). The microdiffusion technique was performed as described in section 10.9.1. Identical results were achieved with IgM and sIgA purified by gel filtration (section 10.9.2; 10.10.2).

Figure 10.6.1. Goat anti-rabbit IgM and anti-rabbit IgG were placed at  $10^{-1}$  dilutions in TBS in wells a and b respectively. A sample of putively purified IgM was placed in the central well, c. A precipitin line can be seen between the centre well and well a.

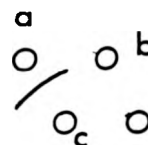
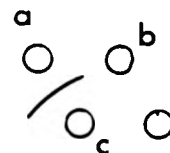


Figure 10.6.2. Goat anti-rat IgA, goat anti-rat IgG and sheep anti-rat IgM were placed at  $10^{-1}$  dilutions in TBS in wells a, b and c respectively. A sample of putively purified sIgA was placed in the central well, d. A precipitin line can be seen between the centre well and well a.



the immunoprecipitin line between anti-rat IgA and the sample well confirming the immunological identity of sIgA.

Purified sIgA obtained using an anti-alpha chain affinity chromatography retained antiviral activity without loss. By non-reducing SDS-PAGE (Figure 10.4), sIgA was shown to consist of a single protein species (Figure 10.6.2) of MW of approximately 400,000, as expected (Koshland, 1975; Tomasi, 1976).

#### 10.10.2. Isolation of sIgA by gel filtration.

Alternatively sIgA was purified on a G-200 Sephadex (Pharmacia, Uppsala, Sweden) column. Firstly, 16ml of crude bile was mixed with 8ml isotonic saline (0.9% w/v NaCl) and centrifuged for 1 h at 95,000g at 4°C. The pale green supernatant was removed and the pellet discarded. The bile/saline mixture was then concentrated ten-fold using centricon microconcentrators. 2ml of the concentrate was applied to the column and eluted with 0.1M sodium phosphate buffer pH6.8 at 4°C. 3ml fractions were collected and monitored by absorbance at 280 nm. Secretory IgA was eluted in the first major peak (Figure 10.5) after a small amount of excluded material. Gel filtration yielded 76 times as much sIgA as affinity chromatography.

An  $E_{1\%}^{1\text{cm}}$  of 13.4 was used to calculate the concentration of IgA (Schultz and Heremans, 1966). Secretory IgA produced by

this method was characterized by SDS-PAGE (5% acrylamide) under non-reducing conditions gave a strong band at 400,000 MW (data not shown) in a similar manner to that achieved with affinity purified sIgA (Figure 10.4). Under reducing conditions (Figure 10.7) sIgA purified by gel filtration was separated into Sc, H $\alpha$  chain and L chain polypeptides. The number of sIgA molecules was calculated using an estimated MW for rat sIgA of 405,000 (Tomasi, 1976). Secretory IgA purified by gel filtration was characterized immunologically by immunodiffusion (giving similar results to those with affinity purified sIgA, Figure 10.6.2)

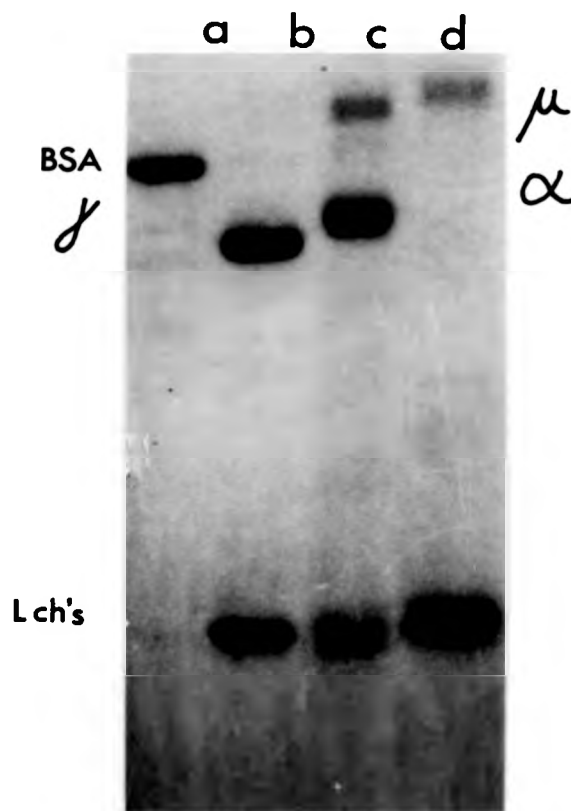
Purified sIgA was stored at - 70°C. Pre-immune sIgA (psIgA) was prepared from bile from non-immunized rats.

#### 10.10.3. Preparation of monomeric IgA from sIgA.

Monomeric IgA was prepared from purified sIgA (obtained by affinity chromatography) by differential reduction with 0.01M beta-mercaptoethanol (BME) (Sigma) in 0.3M Tris-HCl (pH8) for 1h at 25°C. After dialyzing for 18h against 1mM Tris-HCl, 0.015 M sodium chloride (pH7.4), IgA was separated from the secretory component (Sc) and J chain by affinity chromatography on a anti alpha-chain column (section 10.10.1). The J chain and Sc emerged in the void volume and IgA monomers were eluted as before and lyophilized. IgA (160,000 MW) was characterized by PAGE (Figure 10.4) and by immunodiffusion against anti alpha-chain antibody.

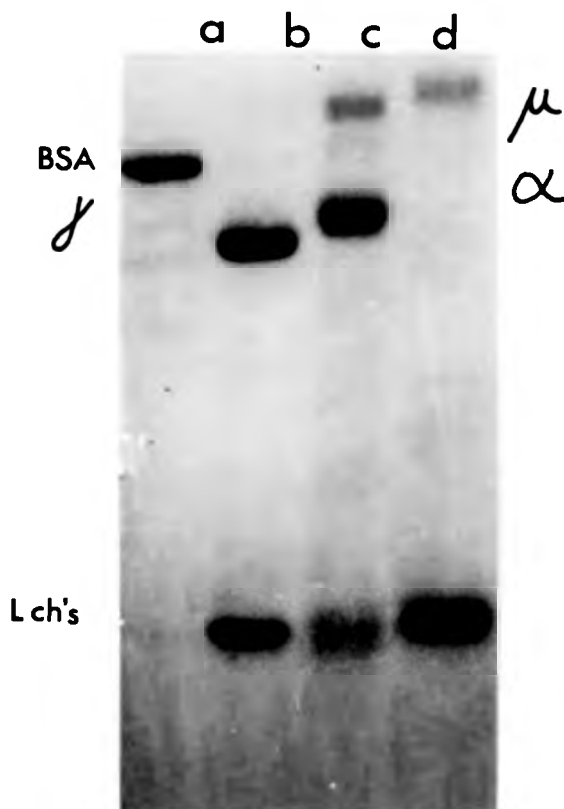
**Figure 10.7. SDS-PAGE analysis of sIgA and IgM purified by gel filtration (section 10.10.2; 10.9.2). The samples were run on the gel (10-30% acrylamide) in reducing solubilizer buffer (section 10.6). The gel was stained for protein with Coomassie Brilliant Blue.**

Lane a BSA (68,000 MW); lane b IgG, gamma H chain (50,000 MW) and L chain (25,000 MW); lane c sIgA, Sc (72,000 MW), alpha H chain (55,000 MW) and L chain (25,000 MW); lane d IgM, mu H chain (75,000 MW) and L chain (25,000). The J chains from both sIgA and IgM were not resolved in this gel system.



**Figure 10.7. SDS-PAGE analysis of sIgA and IgM purified by gel filtration (section 10.10.2; 10.9.2).** The samples were run on the gel (10-30% acrylamide) in reducing solubilizer buffer (section 10.6). The gel was stained for protein with Coomassie Brilliant Blue.

Lane a BSA (68,000 MW); lane b IgG, gamma H chain (50,000 MW) and L chain (25,000 MW); lane c sIgA, Sc (72,000 MW), alpha H chain (55,000 MW) and L chain (25,000 MW); lane d IgM, mu H chain (75,000 MW) and L chain (25,000). The J chains from both sIgA and IgM were not resolved in this gel system.



The HI titre per mg of IgA was never <80% of the titre of the sIgA from which it was derived. J chain (Koshland, 1975) (15,000 MW) was not detected on the gels.

Purified IgA was stored at - 70°C, with BSA (to 0.5mg/ml) if the concentration of IgA was less than 0.1 mg/ml. The neutralization activities of bile concentrate (x 20 fold), affinity purified sIgA, and IgA are shown in Table 10.2. All failed to neutralize influenza A/WSN.

Table 10.2. Neutralization of FPV/R influenza virus by bile concentrate, purified sIgA, and monomeric IgA. Percentage neutralization (section 10.11) was determined relative to virus incubated with the appropriate control (bile concentrate, purified sIgA and monomeric IgA) obtained from non-immunized rats. These gave negligible neutralization.

Immune sample:	Percentage neutralization of:	
	1 x 10 <sup>7</sup> PFU FPV/R	1 x 10 <sup>7</sup> PFU WSN
Bile concentrate	98.5	0
Purified sIgA	96.5	0
Purified monomeric IgA	98.5	0



### 10.11. Neutralization Assays

Influenza virus was incubated with purified immunoglobulins or IgG fragments for 1h at 25°C in a water bath before assaying for residual infectivity on CEF cell monolayers. F(ab')<sub>2</sub> and Fab' fragments neutralized FPV/R with about the same efficiency as the IgG from which they were derived (Table 10.3). However the Fab' underwent dilution dissociation with resulting restoration of virus infectivity. When it was necessary to employ a dilution step to measure the extent of neutralization (i.e. after neutralization of a high titre virus preparation) the virus-Fab' complex was incubated with rabbit anti-mouse IgG (Fab' specific) (Nordic) This stabilized neutralization before inoculation and subsequent assay on cell monolayers.

A neutralization assay was performed in parallel to each attachment and penetration assay with each immunoglobulin investigated (sections 11.1; 11.2) and the results of the neutralization assay is given where pertinent.

In a series of experiments to investigate the importance of polyvalency virus neutralized with IgG was reacted with rabbit anti-mouse IgG (Fc region specific) (RAM) (Nordic). Neutralizing mouse monoclonal IgG (HC2) was incubated with [<sup>32</sup>P]-labelled FPV/R virus for 1h at 25°C and subsequently RAM was added to 5% protein w/v in PBS for a further 1h at 25°C.

Neutralization was also measured by 50% endpoint titration. In this manner the dilution of antibody that reduced a 50-60 PFU FPV/R by 50% was determined. This method was used in the

Table 10.3. Comparison of the efficiency of neutralization<sup>b</sup> by monoclonal anti-haemagglutinin IgG (HC2) and fragments derived thereof.<sup>a</sup>

Antibody/ antibody fragment	Approx. MW (K)	No. of molecules reacted with virus <sup>d</sup>	Total No. of binding sites available	% Neutral- ization
IgG	150	$1 \times 10^{13}$	$2 \times 10^{13}$	99.5
F(ab') <sub>2</sub>	120	$1 \times 10^{13}$	$2 \times 10^{13}$	98.5
Fab'	50	$1 \times 10^{13}$	$1 \times 10^{13}$	98.0

<sup>a</sup> Section 10.8.

<sup>b</sup>  $9 \times 10^7$  PFU (which contains 200 HAU).

<sup>c</sup> Neutralization by incubation with Fab' followed by 5% RAM in PBS; equally efficient neutralization is obtained in conventional neutralization test (section 10.11) providing there is no dilution of the virus-Fab' mixture.

<sup>d</sup> The number of molecules of antibody or antibody fragments was determined using an E 1%/280 nm of 13.5,  $L = 6.02 \times 10^{23}$  /mol and the respective MWs.

investigation of the kinetics of neutralization. Purified monoclonal IgG was incubated with 50-60 PFU at 4 or 25°C for time intervals from 0-45 min before inoculation on to CEF cell monolayers held at 4°C. Incubation on cell monolayers was for 45 min before overlaying with 0.9% agar in 199 medium/5% NCS followed by incubation for 3 days.

#### 10.12. HA-inhibition (HI) assay.

The titres of anti-HA serum, or purified immunoglobulin were determined by making doubling dilutions in 0.25ml volumes of PBS, adding 4 HAU virus to each dilution and incubating for 1h at room temperature. The chicken erythrocytes were added to 0.5% (equivalent to  $7.3 \times 10^8$  erythrocytes per well) and the 50% end-point estimated after 30 min as for the HA assay.

#### 10.13. Iodination of Immunoglobulins.

Antibody was iodinated by a modification of the chloramine T method (Hunter and Greenwood, 1962). Firstly 15  $\mu$ l 100 mM sulphuric acid and 15  $\mu$ l 1.5 mM chloramine T (BDH) were mixed with 15  $\mu$ l potassium iodide and 250  $\mu$ Ci of sodium [ $^{125}$ I]-iodide (Amersham) and left for 30 seconds. After this time 15  $\mu$ l sodium phosphate buffer (pH 7.5) was added, and the mixture was then combined with 500  $\mu$ l antisera containing polyclonal IgG or 500  $\mu$ l

ascites containing monoclonal IgG. This was left for 2 min before being applied to and purified on a protein A sepharose CL-4B column (Sigma) as described in section 10.7. The radioactivity in the IgG peak was measured and the protein concentration determined by absorbance at 280 nm ( $E_{1\%/280} = 13.5$ ). Purified sIgA and IgM were also iodinated using the chloramine T method as described above except that the sodium phosphate buffer contained 0.5mg/ml BSA as a protein carrier for the labelled protein. Radiolabelled sIgA or IgM was separated from unreacted [ $^{125}\text{I}$ ]-iodide on a 0.5 x 10 cm column of Sephadex G-25 (Pharmacia) equilibrated in 0.25M sodium phosphate buffer (pH 7.5) containing 0.5 mg/ml BSA. The column was run until the immunoglobulin and the [ $^{125}\text{I}$ ]-iodide peaks had been eluted. The specific activity of the iodinated antibodies was determined and the biological activities of the radiolabelled immunoglobulins were assayed by HI and neutralization ability.

**10.14. Mapping of monoclonal antibodies to FPV/R to non-overlapping epitopes on the FPV/R haemagglutinin by the isolation of antibody resistant mutants.**

10<sup>8</sup> PFU FPV/R were incubated in turn with each of the monoclonal antibodies (HC2, HC10, HC58 and HC61) for 1h at 25°C. All monoclonal antibodies were at concentrations that would give 99.99% neutralization. Aliquots were injected into 11 day old fertile hens eggs and incubated for 22h at 37°C. Allantoic fluid

was harvested and examined for the presence of putative resistant variants (escape mutants) resistant by HI to the selecting monoclonal antibody. Consistent lack of HI indicated that particular escape mutant was no longer susceptible to that particular antibody. The treated virus was plaque purified then incubated again with the selecting antibody (as above) and passaged through eggs. This was repeated twice in order to obtain stable escape mutants. The resultant HI pattern provided evidence of non-overlapping epitopes to which the monoclonal antibodies bound.

The ability of polyclonal IgG (WR6, WR45 and WR69) to select escape mutants was also investigated in a similar manner. This was necessary to determine if the populations of specific IgG in the sera were recognizing single epitopes (i.e. "pseudo-monoclonal" in nature) or a range of epitopes (i.e. genuinely polyclonal).

#### 10.15. Production and characterization of influenza A/V31/83.

Influenza A/V31/83 (V31) (H7N2) is a reassortant non-pathogenic fowl plague strain derived from a double infection of CEF monolayers with FPV/R and A/X49 (H3N2) conducted according to the method described by Rott et al. (1976). CEF cell monolayers ( $1 \times 10^7$ ) were co-infected with  $3 \times 10^8$  PFU of each virus in 200  $\mu$ l PBS for 1 h at 25°C. The inoculum was then removed and monolayers were incubated for 15 h at 37°C under 3 ml buffered 199 medium. The virus yield was diluted and plated onto confluent CEF cell monolayers and incubated for 3 days under an agar overlay (section 9.1) containing anti-N1 (FPV/R NA) serum at 5% v/v. This concentration of anti-N1 serum suppressed the plaque formation of virus that contained the homologous neuraminidase. Thus the agar selects for virus carrying the H7 HA, against FPV/R NA and against H3 influenza viruses which require trypsin for infectivity (Klenk et al., 1975).

Dishes with not more than 25 plaques were selected as a source of virus clones. The agar and the underlying cells over the area of well-isolated plaques were picked with sterile Pasteur pipettes, added to tubes containing 1ml 5% BSA in PBS and transferred to 11 day old embryonated eggs for the production of virus. Before serological tests and further characterization virus isolates were purified by a further 3 serial plaque passages in the selection media.

The reassortants were then screened for H7 HA with H7 specific monoclonal and polyclonal IgG and for H3 HA with H3 specific monoclonal IgG by HI assay (section 10.12).

Table 10.4. Biological properties of influenza viruses A/FPV/R and reassortant A/V31.

Strain	HA <sup>a</sup>	NA <sup>b</sup>	Plaque diameter	HAU/ml <sup>c</sup>	PFU/ml <sup>c</sup>	Embryo death <sup>d</sup> at 24h pi	Pathogenicity <sup>e</sup>
FPV/R	H7	N1	>2mm	1024	2 x 10 <sup>9</sup>	+	N/A
V31	H7	N2	<1mm	630	3.9 x 10 <sup>8</sup>	-	Alive

<sup>a</sup> As determined by HI assay (section 10.12).

<sup>b</sup> As determined by NI assay (section 10.11).

<sup>c</sup> Virus activity in allantoic fluids 22h after inoculation of 2 x 10<sup>4</sup> PFU virus.

<sup>d</sup> Embryos examined after inoculation with 2 x 10<sup>4</sup> PFU.

<sup>e</sup> Condition of embryos six days post-inoculation.

The neuraminidase was examined by neuraminidase inhibition (NI) assay. NA and NI assays were carried out using fetuin as a substrate. The NA activity of each virus was assayed by incubation of 0.1ml virus with 0.1 ml fetuin (12.5 mg/ml) in sodium phosphate buffer for 1 h at 37°C. Neuraminidase releases N-acetylneuraminic acid from the fetuin which was measured using the colorimetric assay developed by Aminoff (1961). The NI assay measured residual NA activity in virus-antiserum mixtures after incubation at 25 °C for 1 h. Before use in the NI assay non-specific inhibitors were inactivated by the incubation of 1 ml serum with 4 ml 0.1% (w/v) trypsin in sodium phosphate buffer (pH 7.5) for 30 min at 56°C. To this mixture 3 ml (2.6 mg/ml) potassium iodate was added at 4°C for 15 min. After this time 3 ml glycerol was added. The mixture was dialysed for 18 h against PBS at 4°C before being used in the assay.

To test for virulence and pathogenicity 11 day old embryonated hens eggs were inoculated with  $2 \times 10^4$  PFU virus. At 24 and 48 h post-inoculation the condition of the embryos and the allantoic fluid were examined. Embryos were also examined 6 days post inoculation for development. The pathogenic effect produced by V31 was minimal; embryos inoculated with V31 were comparable to appearance to control embryos. Those inoculated with FPV/R were dead after six days.

On the isolation of the desired reassortment after the above



assays a further 3 plaque purification cycles were performed. Table 10.4 classifies the biological properties of V31 compared to parental strains.

11.1. Assay for attachment of neutralized and non-neutralized [ $^{32}$ P]-labelled FPV/R to avian and mammalian cells.

Virus was first incubated for 1 h at 25°C with neutralizing immunoglobulin, bile concentrate, IgG fragments or the equivalent from preimmune sources. 100ul volumes of virus-Ig mixtures were inoculated onto monolayers of BHK-21 cells in plastic Petri dishes, held on ice to give a temperature at the monolayer surface of 4°C, unless otherwise stated. This temperature prevents virus directed macromolecular synthesis (Stephenson and Dimmock, 1975). At the required times monolayers were rinsed three times with cold PBS and removed by scraping into 0.5 ml cold PBS. Radioactivity associated with cells and washes was determined by binding to Whatman DE81 filter discs (Whatman Ltd., Maidstone U.K.), which were washed successively with sodium dihydrogen orthophosphate (three times), water, and ethanol. Filter papers were inserted into scintillation vials containing a pre-mixed liquid scintillation cocktail, Ready Solv EP (Beckman Instruments, Galway, Ireland). The amount of radioactivity associated with the cells was determined using a Packard Tri-carb (model 3385) Liquid scintillation spectrometer. The radioactivity determined represented [ $^{32}$ P]-labelled virion RNA. Lipids and

proteins do not bind to Whatman DE81 filter paper (Whatman Ltd. Technical Enquiry Service). This was confirmed by the evidence that [ $^{125}\text{I}$ ]-labelled antibody does not bind. Results were expressed as a percentage of the [ $^{32}\text{P}$ ]-labelled virion RNA originally inoculated.

The majority of experiments were carried out on BHK-21 cells, but the ability of neutralizing IgG to interfere with the binding of FPV/R to Ls29, HFF-4, and CEF cells was also investigated.

#### 11.2. Assay for the uptake of virion RNA into the nuclei (using a nuclear monolayer fractionation technique).

BHK-21 cells were fractionated by removing cytoplasm from cell monolayers by an adaptation of the technique described by Hudson and Dimmock (1977). BHK-21 cells were incubated for two successive periods of 5 min, in 0.5% Nonidet P-40 (NP-40) (BDH Pharmaceuticals Ltd. London, U.K.) in 0.25 M sucrose, 0.01 M Tris-HCl (pH 7.4), 1 mM  $\text{MgCl}_2$  on ice. Nuclei remained attached to the Petri dish and were scraped into the same buffer without NP-40, rinsed vigorously on a vortex mixer for 60 sec, and then pelleted at 150 g for 5 min at 4°C. This last step is crucial to remove all remaining cytoplasmic tags. The supernatant was pooled with the cytoplasmic fraction. This method gives a high yield of nuclei that have a cytoplasmic contamination of approximately 5%

(section 11.2.1). Uptake of [ $^{32}\text{P}$ ]-labelled virion RNA was measured by inoculating monolayers with neutralized or non-neutralized virus and fractionating the cells at intervals. Radioactivity was collected as before (section 11.1) using Whatman DE81 filters. This procedure provides a measure of the early stages of infection up to the transport of virion RNA to the nucleus. (Hudson and Dimmock, 1977). The same technique was performed for the fractionation of CEF cells except that 2% NP-40 was used (Hudson and Dimmock, 1977).

#### 11.2.1. Validation of the nuclear monolayer fractionation methodology.

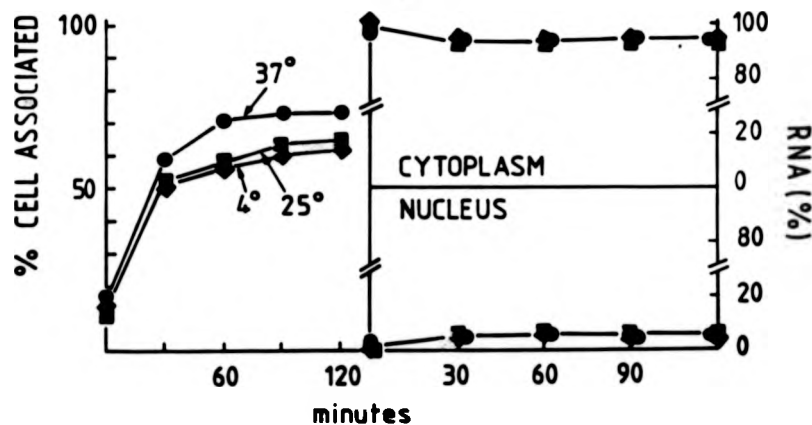
To test the possibility that there was plasma or other membrane contamination of nuclei, BHK-21 cell monolayers were inoculated with  $6 \times 10^5$  PFU [ $^{32}\text{P}$ ]-labelled SFV ( $6 \times 10^4$  CPM). SFV multiplies exclusively in the cytoplasm (Grimley et al., 1968) and therefore no RNA should be detected in the nuclear fractions of BHK-21 cells. The nuclear fractionation assay was carried out with SFV as described in section 11.2. Fig 11.1.1 shows the rate of attachment at 4, 25 and 37°C and Fig 11.1.2 shows that irrespective of temperature 95% of SFV remains in the cytoplasm.

The assay was also validated by assay for HA production in the cytoplasm of infected cells, uptake of [ $^3\text{H}$ ]-thymidine in the

Figure 11.1.1 and Figure 11.1.2. Validation of the nuclear monolayer fractionation method using BHK-21 cell monolayers ( $1 \times 10^7$  cells) inoculated with purified [ $^{32}$  P]-labelled Semliki Forest virus (SFV). Monolayers were inoculated with  $6 \times 10^5$  PFU containing  $6 \times 10^4$  cpm with replicates at 4, 25 and 37°C. Radiolabelled virion RNA binding to Whatman DE 81 filter discs (section 11.1) is presented here. After incubation monolayers were washed extensively with PBS and all placed at 4°C. Nuclei were isolated using the method described in section 11.2. Figure 11.1.1 represents virus attached to cells and Figure 11.1.2 represents the distribution of virion RNA between the cytoplasmic and nuclear fractions.

Figure 11.1.1

Figure 11.1.2.



nuclei, uptake of [ $^3$  H]-uridine in the cytoplasm and monitored by phase contrast microscopy. The HA of influenza in infected cells remains cytoplasmic. Therefore removal of HA from the periphery of the nucleus would provide a stringent test of purity. To assay for HA production in BHK-21 cells, monolayers were inoculated with 100  $\mu$ l PBS containing  $10^7$  PFU FPV/R for 1h at room temperature, the inoculum washed away before incubation for a further 5h at 37°C in 3 ml GMEM/5% NCS. Cells were then fractionated as previously described or scraped into 0.5 ml PBS. The nuclei and whole cell fractions were assayed for HA. BHK-21 cell monolayers were incubated for 24h at 37°C with 3 ml GMEM/5%NCS containing either 4  $\mu$ Ci [ $^3$ H] thymidine (Amersham) to label DNA in the nuclei or 4  $\mu$ Ci [ $^3$ H] uridine (Amersham) to label RNA in the cytoplasm. Cells were chased for 24h with medium without radioisotope then fractionated as previously described. Radioactivity in the cytoplasmic and nuclear fractions was determined. Parallel experiments were performed on CEF cells.

Table 11.1 summarizes the results obtained using the fractionation method on BHK-21 cell monolayers showing that little newly synthesized HA was associated with the nuclei (2.2%), labelled DNA was mostly nuclear (7.7%) and labelled RNA was cytoplasmic (92%). On the basis of the results obtained with SFV it is assumed that the 5% of virus RNA in the nucleus of BHK-21 cells represents the level of contamination by cytoplasmic constituents.

Table 11.1. Summary of the assessment of the nuclear monolayer fractionation method for obtaining nuclei from BHK-21 cell monolayers. Duplicate monolayers were: (a) Inoculated with  $10^7$  PFU FPV/R for 1 h at  $25^\circ\text{C}$  followed by 5 h at  $37^\circ\text{C}$  in 3ml 199 medium. Cells were then fractionated as described (section 11.2) or scraped into 0.5 ml PBS. Cells were then assayed for HA. (b) Incubated for 24 h with 4  $\mu\text{Ci}$  [ $^3\text{H}$ ]-thymidine or (c) 4  $\mu\text{Ci}$  [ $^3\text{H}$ ]-uridine in 3ml 199 medium at  $37^\circ\text{C}$  then chased with medium without radiolabel for 24 h. Cells were then fractionated as described (section 11.2). (d) Inoculated with  $6 \times 10^5$  PFU [ $^{32}\text{P}$ ]-labelled SFV containing  $6 \times 10^4$  cpm (section 10.3.1) at  $25^\circ\text{C}$  for 120 min (Figure 11.1.2). Cells were then fractionated as described (section 11.2) and radioactivity determined by binding to Whatman DE81 filter discs. The corresponding percentage value obtained in the nuclei of CEF cell monolayers ( $10^7$  cells) treated in a similar manner is shown. The nuclear monolayer fractionation of CEF cell monolayers requires 2% NP40 (Hudson and Dimmock, 1977).

Assay	Cell assoc.	Cytoplasm (%) (cpm)	Nucleus (%) (cpm)	% in CEF nuclei
(a) HA (HAU)	3548 HAU	-	-	3
(b) DNA [ $^3\text{H}$ ]	-	10436 (9)	105270 (91)	94
(c) RNA [ $^3\text{H}$ ]	-	160620 (92.3)	13329 (7.7)	10
(d) SFV [ $^{32}\text{P}$ ]	-	34570 (95)	1820 (5)	6

Phase contrast microscopy showed that BHK-21 nuclei were discrete refractile bodies without any cytoplasmic "tags" after fractionation. Possee (1981) had previously shown that the nuclear monolayer technique produces nuclei comparable to those produced by Dounce homogenization. The nuclear monolayer fractionation technique has the advantages of minimizing damage to nuclei and of allowing several monolayers to be fractionated simultaneously and speedily.

#### 11.3. Proteinase K removal of [ $^{32}$ P]-labelled FPV/R attached to the surface of BHK-21 cells.

To determine the percentage of virus attached to BHK-21 cells, [ $^{32}$ P]-labelled FPV/R-antibody mixtures were incubated with monolayers for 2h, and cells were then rinsed three times with cold PBS and incubated at 4°C with 2  $\mu$ g/ml proteinase K (sp. act., 20 Anson units/g; BDH) in 500 $\mu$ l. After 60 min, the enzyme solution was removed and pooled with two subsequent washes of 250  $\mu$ l PBS. The cells were then fractionated and radioactivity determined as before. The proteinase K was auto-digested before use by incubation of 0.5  $\mu$ g/ml in PBS for 1 h at 37°C.

##### 11.3.1. Validation of the proteinase K removal technique.

0.1 mg concanavalin A (Sigma) in 400  $\mu$ l PBS was iodinated

with [ $^{125}$ I]-iodine using the chloramine T method (section 10.13.). 100  $\mu$ l PBS containing 5  $\mu$ g [ $^{125}$ I]-labelled concanavalin A (38410 CPM) was inoculated onto BHK-21 cell monolayers. After 30 mins cells were washed three times with cold PBS. The cell monolayers were then incubated with 2  $\mu$ g/ml proteinase K for 60 min at 4°C. This was found to remove 85% of the [ $^{125}$ I]-labelled concanavalin A associated with an untreated BHK-21 cell monolayer.

#### 11.4. Attachment of radiolabelled antibody to FPV/R particles.

Antibody-virus mixtures were initially used at the same ratio as previously used in virus-cellular association studies; that is the amount of polyclonal IgG that gave 99.95% neutralization of  $1 \times 10^7$  PFU or 3.75 haemagglutination inhibition units (HIU) of purified antibody to 1 HAU virus. The standard assay conditions were as follows: iodinated immunoglobulin was incubated with 20 HAU FPV/R plus 20  $\mu$ g cytochrome C for 1 h at 25°C. The mixtures were made up to 300  $\mu$ l total volume with PBS. At the end of this time the mixtures were incubated for 18h at 4°C to allow equilibration. Virus-[ $^{125}$ I]-labelled antibody complexes were sedimented through a 5 ml cushion of 5% (w/v) sucrose in PBS by ultracentrifugation at 75,000g for 90 min in BSA coated 5.5ml polycarbonate tubes. This separated complexes from free antibody. The supernatants were discarded and the amount of radioactivity associated with



the pellet determined. Iodinated IgG binding to FPV/R was determined with respect to nonspecific binding to influenza B/Lee.

#### 11.4.1. Two step competition binding assay.

This assay consisted of incubating 20 HAU FPV/R particles with two different antibodies sequentially. Firstly unlabelled immunoglobulin was incubated with 20 HAU virus 1h at 25°C then for 18h at 4°C then the radiolabelled second antibody was added, incubated as before, and the virus and any associated antibody was pelleted. Ratios of unlabelled and [ $^{125}$ I]-labelled antibody were varied in order to investigate the amount of binding that could be achieved by the labelled antibody.

#### 11.4.2. Solid phase radioimmunoassay (RIA)

A solid phase radioimmunoassay system was developed to check the results obtained from the competitive binding of virus and antibody in solution. Competitive binding RIAs were performed on viral antigens adsorbed onto the wells of polyvinyl flat bottomed 48 well tissue culture plates (Linbro Scientific, subsidiary of Flow Laboratories). 5 HAU of purified FPV/R in 100ul PBS (0.1% BSA) were adsorbed onto each well of the microtitre plate by incubating for 18h at 4°C. Wells were washed 3 times with PBS

before use in the assay.

Haemagglutinin was cleaved from FPV/R by treatment with bromelain (Grade II) (Sigma Chemical Co.) as detailed by Brand and Skehel (1972).  $3 \times 10^4$  HAU FPV/R (approx. 80  $\mu$ g) was incubated for 16 h at 37°C with 40  $\mu$ g bromelain in 0.1 M Tris/HCl (pH 8) and 0.05 M beta mercaptoethanol (total volume equalled 200 $\mu$ l). After digestion the virus "cores" and undigested virions were sedimented through a 5 ml cushion of 5% (w/v) sucrose in TBS by ultracentrifugation at 75,000g for 90 min at 4°C. Cleaved haemagglutinin (BrHA) did not enter the sucrose cushion to any extent and the top 1 ml was dialyzed against 3 changes of PBS over 10 h. BrHA was passively adsorbed onto the wells of titre plates for 6h at 25°C, then for 12h at 4°C (Gerhard et al., 1981) before assay for HA activity.

The assay consisted of incubating mixtures of antibody and iodinated antibody with viral antigen coated wells for 1h at 25°C and 18h at 4°C. Then wells were thoroughly washed with cold PBS and the radioactivity associated with the wells determined.

## RESULTS AND DISCUSSION

## SECTION 12:

MECHANISMS OF NEUTRALIZATION OF  
INFLUENZA VIRUS A/FOWL PLAGUE/ROSTOCK/34 (H7N1)

## Summary

The neutralizing activity of various immunoglobulins and IgG fragments with specificity for the haemagglutinin of type A influenza virus (A/Fowl plague virus/Rostock/34; H7N1) (FPV/R) were investigated. When neutralized by either IgG or monomeric IgA, FPV/R attached to and penetrated into BHK-21 cells, at a rate indistinguishable from that of non-neutralized virus. This behaviour was independent of temperature from 4 to 37°C. Thus IgG and monomeric IgA neutralized infectivity at a stage subsequent to penetration. Virus was efficiently neutralized by F(ab')<sub>2</sub> and Fab' fragments of monoclonal anti-HA IgG. Neither prevented the attachment, penetration, or the accumulation of viral genomes in the nucleus.

In contrast the polymeric immunoglobulins secretory IgA (sIgA) and IgM completely blocked attachment at 4°C. The large molecular structure of these antibodies suggests that steric hindrance may be the mechanism by which attachment is impaired. However at 25 and 37°C neutralizing sIgA and IgM inhibited attachment of only 50% of the neutralized virus but none of this attached virus was internalized. Therefore it seems likely that IgM and sIgA interfere with the endocytotic event responsible for internalization of virus.

### Introduction.

Infection is initiated by attachment of virus to cells and it has been assumed that neutralizing antibody acts by combining with the virus attachment protein and preventing this process. This generalization, however, is not always true (section 9.2.1; section 9.2.2; Joklik, 1964; Mandel, 1967; Yoden et al., 1982; Possee et al., 1981; Emini et al., 1983a; Schlegel and Wade, 1983; Kingsford and Ishizawa, 1984; Fuller and Spear, 1985), and it is emerging through studies such as these that there are mechanisms other than preventing attachment by which virus is neutralized (Dimmock, 1984).

It has been found previously (Possee et al., 1981; Possee, 1981) that FPV/R neutralized by polyclonal or monoclonal IgG attached to chick embryo fibroblast (CEF) cells at the same rate as non-neutralized virus. Using a fractionation technique to isolate nuclei from CEF cells, it was found that neutralization of influenza by IgG did not effect the penetration or transport of virion RNA into the nuclei. In fact the rate of nuclear accumulation of [ $^{32}$ P]-labelled RNA from neutralized virus was similar to that of non-neutralized virus.

In this section these earlier studies have been extended to include observations on the neutralization of FPV/R by IgG in BHK-21 cells and other mammalian cells, and investigations on the interactions of FPV/R neutralized by IgM, sIgA, monomeric IgA and IgG fragments with BHK-21 cells. A comparison of the manner in which these antibodies neutralize can now be made.

### 12.1. Mechanisms of neutralization by IgG.

Figure 12.1.1 confirmed previous studies (Possee and Dimmock, 1981; Possee, 1981) that the kinetics of attachment of FPV/R neutralized by IgG to CEF cell monolayers is similar to that of non-neutralized virus. Further to these observations the ability of neutralizing IgG to interfere with the binding of FPV/R to mammalian cells was examined (Figures 12.1.2 - 12.1.4). Serum containing neutralizing IgG did not significantly alter the rate of attachment of virus to mouse (L<sub>12</sub>10), human (HFF-4) and hamster (BHK-21) cells at 4°C. FPV/R, neutralized by purified monoclonal IgG also attached to BHK-21 cells at 4°C at a rate indistinguishable from that of non-neutralized virus (Figure 12.2).

After attachment to the cell, virus is internalized and uncoated; the virus envelope components remain in the cytoplasm while the transcriptase complex of virion RNA and associated proteins enter the nucleus (Stephenson and Dimmock, 1975).

The nuclear monolayer fractionation method (sections 11.2; 11.2.1) was used to determine the proportion of cell-associated virion RNA that enters the nuclei. Validation of this method (section 11.2.1) demonstrated that irrespective of temperature 95% of SFV RNA remains cytoplasmic. It is assumed that the 5% of virion RNA in the nuclear fraction represents the level of contamination by cytoplasmic constituents (Figure 11.1.2).

Figures 12.3 and 12.4 show that the accumulation of RNA from FPV/R neutralized by either polyclonal IgG or monoclonal IgG is

Figures 12.1.1 to 12.1.3 Attachment of neutralized (●) and non-neutralized (◆) [ $^{32}$ P]-labelled FPV/R to CEF cell monolayers (Figure 12.1.1), to L<sub>929</sub> cell monolayers (Figure 12.1.2) and to HFF 4 cell monolayers (Figure 12.1.3).

$2 \times 10^7$  PFU (containing 200 HAU and  $1.7 \times 10^5$  cpm) were incubated with a 1:10 dilution of polyclonal anti HA (●) (rabbit anti A/FPV/D; WR45) or the equivalent pre-immunization serum (pIg) (◆) for 1h at 25°C. Mixtures were then cooled to 4°C and inoculated onto monolayers\* held at 4°C. At each time interval monolayers were rinsed (x3) with cold PBS and the radioactivity associated with cells and in washes was determined by binding to Whatman DE 81 filter discs (section 11.1). This is virion RNA. Neutralization was > 99.9%. Pre-immune serum did not neutralize.

\* CEF and L<sub>929</sub> cell monolayers at  $9 \times 10^6$  cells per dish and HFF 4 cell monolayers at  $1 \times 10^7$  cells per dish.

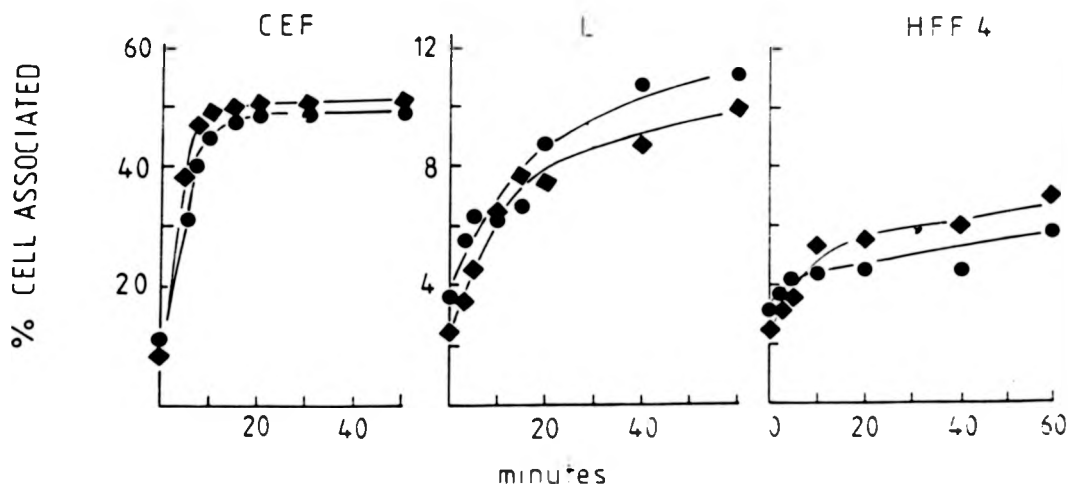




Figure 12.1.4. Attachment of neutralized (●) (N) and non-neutralized (◆) (treated with pre-immune IgG, pIg) [ $^{32}$ P]-labelled FPV/R on BHK-21 cell monolayers at 4°C ( $6 \times 10^6$  cells per plate). Conditions as detailed in Figure 12.1.1.

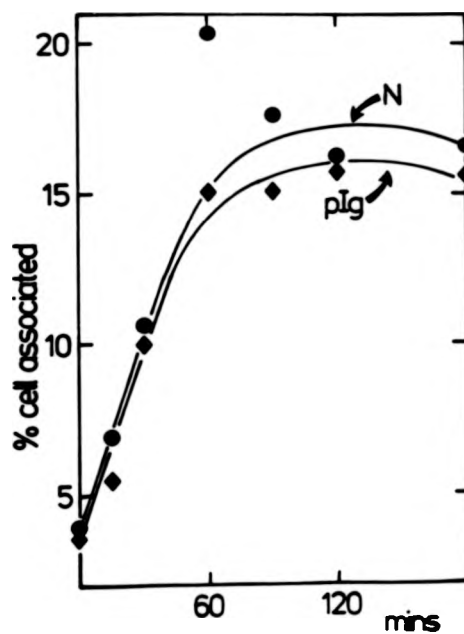


Figure 12.2. Attachment of neutralized (●) and non-neutralized (◆) [ $^{32}$ P]-labelled FPV/R on BHK-21 cell monolayers at 4°C ( $6 \times 10^6$  cells per plate). Conditions as detailed in Figure 12.1.1 except that purified monoclonal anti-H7 IgG (HC2) (section 10.7) was used. Neutralizing IgG reduced the infectivity of  $1.4 \times 10^7$  PFU ( $630$  HAU and  $1.7 \times 10^5$  cpm) by 99.9 %.

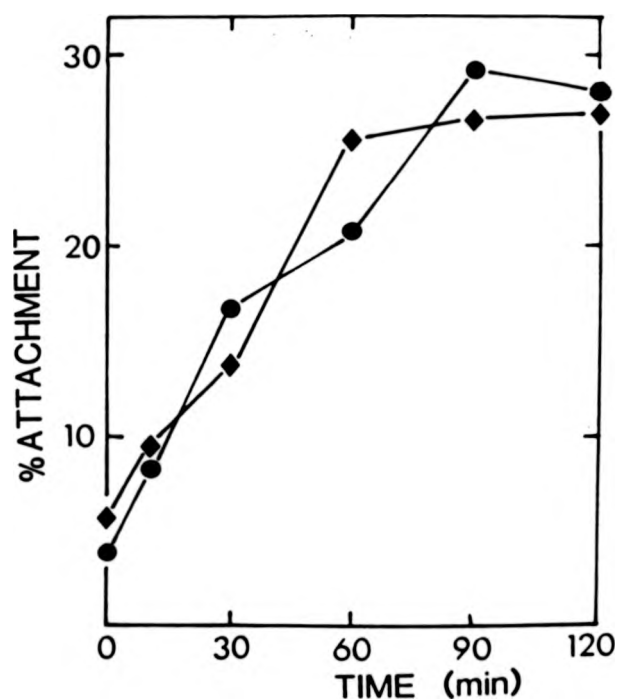


Figure 12.3. Association of non-neutralized (treated with pIg) and neutralized (N) [ $^{32}$ P]-labelled FPV/R with cytoplasmic and nuclear fractions of BHK-21 cells at 4°C. Virus was treated and inoculated as described in Figure 12.1.1. At the intervals indicated nuclear and cytoplasmic fractions were prepared (section 11.2; Figure 11.1.2).

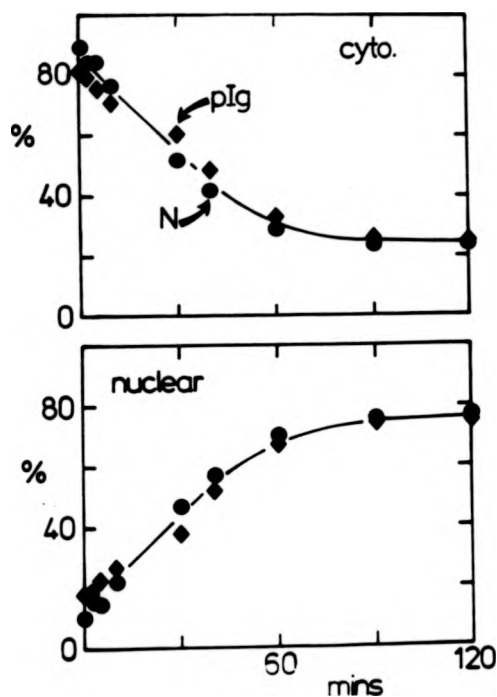


Figure 12.4. Association of non-neutralized and neutralized [ $^{32}$ P]-labelled FPV/R with cytoplasmic and nuclear fractions of BHK-21 cells at 4°C. Virus was neutralized with purified monoclonal anti H7 IgG (HC2) (section 10.7) and inoculated as described in Figure 12.1.1. Neutralizing monoclonal IgG reduced the infectivity of  $1.4 \times 10^7$  PFU (630 HAU and  $1.7 \times 10^5$  cpm) by > 99.9%. At the intervals indicated nuclear and cytoplasmic fractions were prepared (section 11.2; Figure 11.1.2).

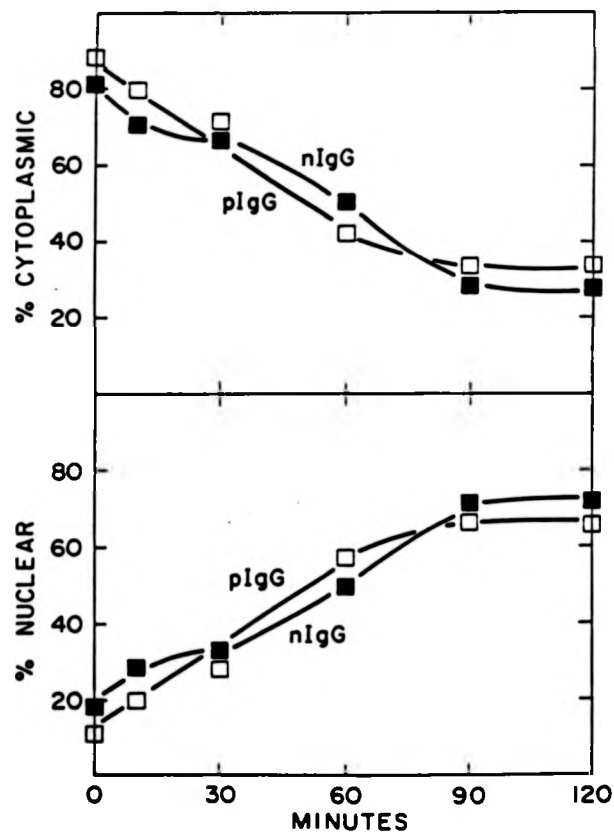


Table 12.1. Summary of the attachment to BHK-21 cells, and association with the nuclear compartments of such cells by [ $^{32}$ P]-labelled RNA from neutralized or non-neutralized FPV/R. Virus was either neutralized by one of a panel of four purified monoclonal IgGs (HC2; HC10; HC58; HC61) or polyclonal IgG (WR69; WR45). Virus-immunoglobulin mixtures total volume = 100  $\mu$ l) were incubated for 120 min on BHK-21 cell monolayers at 4°C before the assays were carried out. 825 HI units (HIU) of each neutralizing IgG was present in each volume giving a HAU:HIU ratio of 3.75.

Purified IgG	% Neutraliz- ation of 1.5 x 10 <sup>7</sup> PFU (220 HAU).	Attachment(%)	Accumulation in nucleus(%)
HC2	99.99	27	69
HC10	99.9	31	72
HC58	99.5	25	76
HC61	99.99	21	68
77/1	-	30	75
WR69	99.8	26	72
WR45	99.5	32	71
pIgG	-	28	68

similar to that of infectious virus. The loss of infectivity due to neutralization by IgG must result from the inhibition of a stage subsequent to penetration.

A summary of several experiments concerning the attachment and association of FPV/R neutralized by IgG with BHK-21 cells compared to that of infectious virus is presented in Table 12.1. The effect of each of four neutralizing monoclonal IgGs was examined; HC2, HC10, HC58 and HC61. All, despite substantial neutralization titres (from 99.5 - 99.99%), failed to inhibit attachment to BHK-21 cells or penetration of the virion RNA into the nuclei of these cells. Attachment and nuclear accumulation of neutralized virion RNA was consistently similar to that of non-neutralized virus. Interaction by FPV/R neutralized by either of the two polyclonal IgGs tested (WR45 and WR69) also confirmed that neutralizing IgG has no effect on attachment or on the stages of infection leading to the accumulation of virion RNA in the nucleus.

Initial experiments used monolayers held at 4°C, as this temperature allows the genome of influenza to enter the nuclei but prevents any virus-directed macromolecular synthesis from taking place (Stephenson and Dimmock, 1975). Subsequent experiments using cells held at 25 and 37°C (data not presented in full, but see Table 12.2) produced results similar to those observed at 4°C.

## 12.2. Mechanisms of neutralization by secretory IgA and monomeric IgA.

FPV/R that had been neutralized with immune bile concentrate (section 10.10) did not attach in significant amounts to BHK-21 cells (Figure 12.5). Secretory IgA was purified from the bile concentrate (section 10.10.1). The attachment to BHK-21 cells at 4°C of virus neutralized by sIgA was compared with virus neutralized by IgG (Figure 12.6). Pre-immune sIgA did not restrict the attachment of virus to the BHK-21 cell monolayers, but attachment of virus neutralized by sIgA (to 96.5%) remained at a low level. In contrast virus neutralized by IgG attached to the cells (section 12.1).

To determine if the ability of neutralized virus to attach to cells is related to the structure of the neutralizing immunoglobulin, the attachment to BHK-21 cells of virus neutralized with monomeric IgA (prepared from sIgA; section 10.10.3) was investigated. Figure 12.7 shows that monomeric IgA, like IgG, did not inhibit attachment. Thus, inhibition of attachment by sIgA appears to be related to its dimeric structure, or this structure plus Sc, and not to any intrinsic property of the immunoglobulin alpha heavy chain itself.

To investigate if virus neutralized with sIgA was able to undergo any of the stages of infection subsequent to attachment (as it does when neutralized with IgG), the levels of virion RNA

similar to that of infectious virus. The loss of infectivity due to neutralization by IgG must result from the inhibition of a stage subsequent to penetration.

A summary of several experiments concerning the attachment and association of FPV/R neutralized by IgG with BHK-21 cells compared to that of infectious virus is presented in Table 12.1. The effect of each of four neutralizing monoclonal IgGs was examined; HC2, HC10, HC58 and HC61. All, despite substantial neutralization titres (from 99.5 - 99.99%), failed to inhibit attachment to BHK-21 cells or penetration of the virion RNA into the nuclei of these cells. Attachment and nuclear accumulation of neutralized virion RNA was consistently similar to that of non-neutralized virus. Interaction by FPV/R neutralized by either of the two polyclonal IgGs tested (WR45 and WR69) also confirmed that neutralizing IgG has no effect on attachment or on the stages of infection leading to the accumulation of virion RNA in the nucleus.

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To investigate if virus neutralized with sIgA was able to undergo any of the stages of infection subsequent to attachment (as it does when neutralized with IgG), the levels of virion RNA

Figure 12.5. Attachment of non-neutralized [ $^{32}$ P]-labelled FPV/R and virus neutralized with immune bile concentrate to BHK-21 cell monolayers at 4°C. Virus was first incubated for 1 h at 25°C with immune bile concentrate ( $\blacktriangle$ ) or the equivalent from non-immunized rats ( $\bullet$ ). Infectivity was neutralized only by the former to 97.5%. The subsequent procedure is described in Figure 12.1.1. except that each 100  $\mu$ l chilled virus-bile mixture inoculated onto the monolayers of BHK-21 cells ( $6 \times 10^6$  cells/dish) contained  $1.1 \times 10^7$  PFU, 320 HAU and  $1.98 \times 10^5$  cpm.

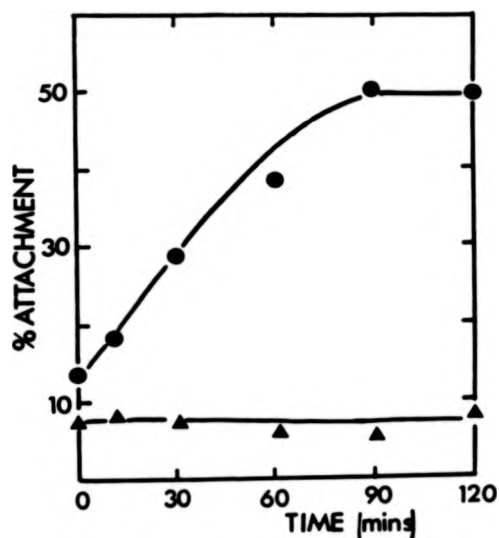
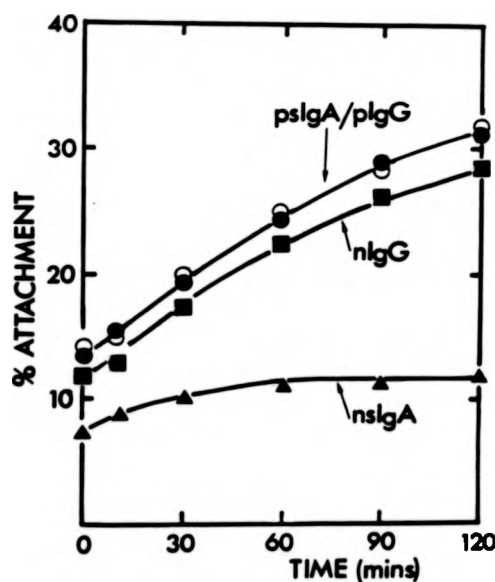


Figure 12.8. Attachment of non-neutralized [ $^{32}$ P]-labelled FPV/R and virus neutralized with purified sIgA to BHK-21 cell monolayers at 4°C. Virus was first incubated for 1 h at 25°C with neutralizing sIgA (nsIgA), neutralizing (polyclonal) IgG (nIgG), pre-immune sIgA (psIgA), or pre-immune IgG (pIgG). Immune sIgA and IgG neutralized virus by 96.5% and 99.5% respectively. the subsequent procedure is described in Figure 12.1.1, except that each 100  $\mu$ l inoculated onto the cell sheet contained initially  $1.2 \times 10^7$  PFU, 36 HAU and  $1.2 \times 10^5$  cpm.



which accumulated in the nuclei were measured.

Experiments on the small fraction of virus neutralized by sIgA that attaches to cells at 4°C (Figure 12.8) showed that virion RNA remained in the cytoplasmic fraction whereas that from virus neutralized by IgG entered the nucleus as previously seen (Figures 12.3; 12.4). It was therefore of particular interest to determine if monomeric IgA, which had no effect on attachment of neutralized virus (Figure 12.7), prevented internalization like sIgA or behaved like IgG. Figure 12.9 shows that RNA from virus neutralized by monomeric IgA accumulates in the nucleus at the same rate as RNA from non-neutralized virus.

Although it is experimentally convenient to examine the early stages of infection at 4°C (since this temperature prevents virus macromolecular synthesis) it is possible that virus may behave differently at physiological temperatures. Fig 12.10 shows that, although sIgA almost completely prevented attachment of neutralized virus at 4°C, attachment at 25 and 37°C was only reduced by 50%. However, in contrast to FPV/R neutralized by IgA or IgG, the majority of virion RNA that associated with the cells at 25 and 37°C remained in the cytoplasmic fraction (Figure 12.11) and less than 5% was taken up into the nuclei.

The cytoplasmic fraction obtained by fractionation includes the plasma membrane and as such it was not possible to determine if virus was attached to the outside of the cell or had been

Figure 12.7. Attachment of non-neutralized [ $^{32}$ P]-labelled FPV/R and virus neutralized with monomeric IgA to BHK-21 cell monolayers at 4°C. Virus was first incubated for 1 h at 25 °C with neutralizing IgA (nIgA), neutralizing IgG (nIgG), pre-immune IgA (pIgA) or pre-immune (pIgG). Immune IgA and IgG neutralized virus by 98.5% and 99.5% respectively. The subsequent procedure is described in Figure 12.1.1, except that each 100  $\mu$ l inoculated onto the cell monolayers contained initially  $1.3 \times 10^7$  PFU, 47 HAU and  $2 \times 10^5$  cpm.

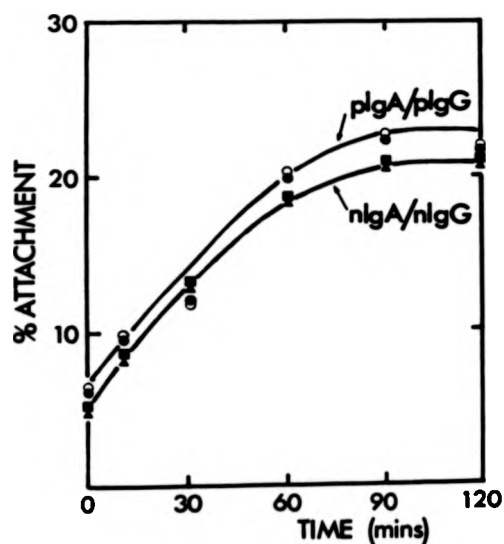


Figure 12.8. Association of non-neutralized and neutralized [ $^{32}$ P]-labelled FPV/R with cytoplasmic and nuclear fractions of BHK-21 cells at 4°C. Virus was neutralized with neutralizing sIgA (nsIgA) or neutralizing IgG (nIgG) and inoculated as previously described in Figure 12.1.1. nsIgA and nIgG neutralized virus by 96.5% and 99.5% respectively. Each 100  $\mu$ l inoculated contained initially  $1.2 \times 10^7$  PFU, 36 HAU and  $1.2 \times 10^5$  cpm. at the intervals indicated nuclear and cytoplasmic fractions were prepared (section 11.2; Figure 11.1.2).

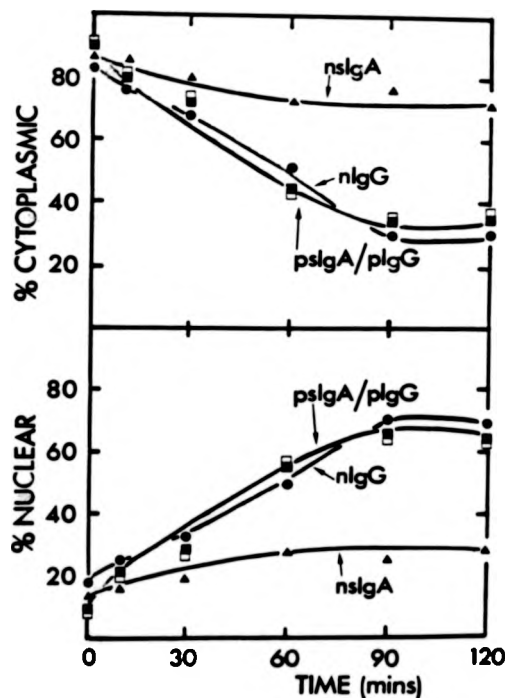


Figure 12.9. Kinetics of migration of virion RNA from cytoplasm to nucleus in cells inoculated with virus neutralized by monomeric IgA and non-neutralized virus at 4°C. Virus was neutralized and inoculated as previously described in Figure 12.1.1. Immune IgA (nIgA) neutralized virus by 98.5%. Each 100  $\mu$ l inoculated initially contained  $1.3 \times 10^7$  PFU, 47 HAU and  $1.2 \times 10^5$  cpm. At the intervals indicated nuclear and cytoplasmic fractions were prepared (section 11.2; Figure 11.1.2).

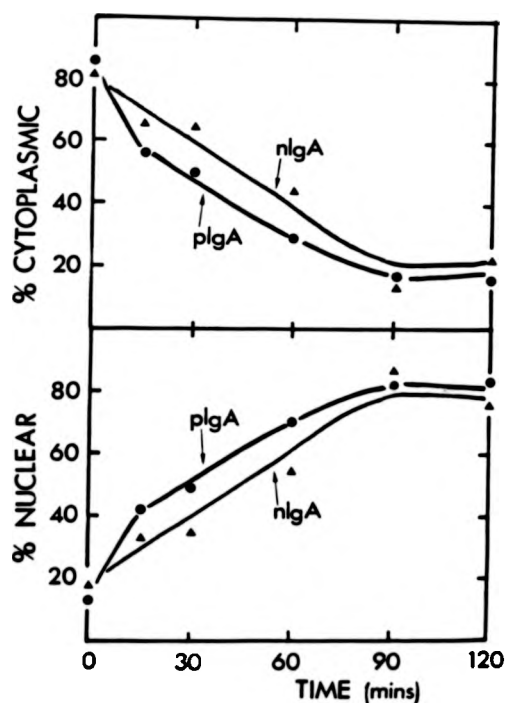


Figure 12.10. Attachment of non-neutralized and neutralized [ $^{32}$ P]-labelled FPV/R to BHK-21 cell monolayers at 4, 25, and 37°C. Virus was first incubated for 1 h at 25°C with neutralizing sIgA (solid symbols) or pre-immune sIgA (open symbols). The neutralizing sIgA reduced infectivity by 98%. Virus antibody mixtures (100  $\mu$ l) were then inoculated onto BHK-21 cells at 4, 25 and 37°C. Each 100  $\mu$ l initially contained  $1.3 \times 10^7$  PFU, 316 HAU and  $1.9 \times 10^5$  cpm. The percentage attachment was determined as previously described.

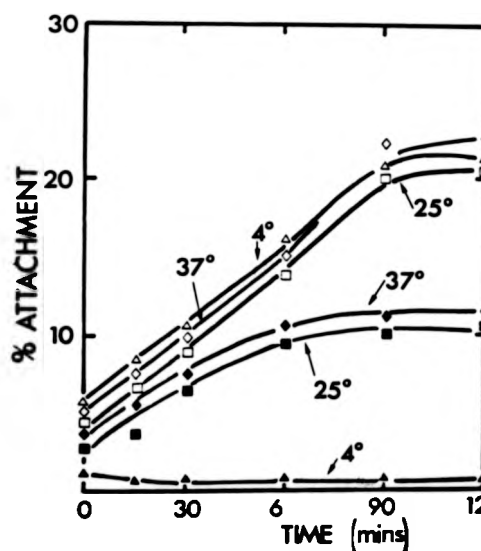
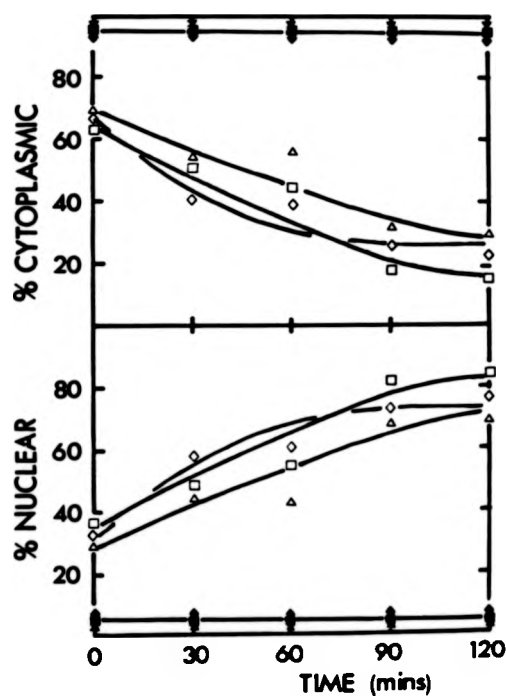
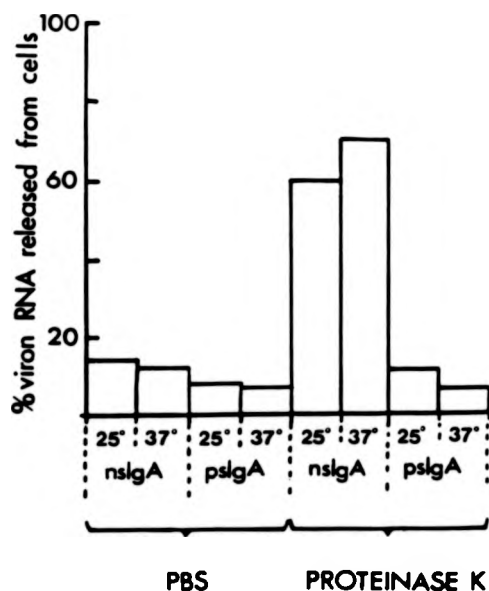




Figure 12.11. Kinetics of migration of [ $^{32}$ P]-labelled virion RNA from cytoplasm to nucleus in BHK-21 cells inoculated with virus neutralized by sIgA. Virus was neutralized and inoculated at 4, 25 and 37°C as described in Figure 12.10. The immune sIgA neutralized virus by 98%. Each 100  $\mu$ l initially contained  $1.3 \times 10^7$  PFU, 316 HAU and  $1.9 \times 10^5$  cpm. Neutralizing sIgA at 4°C ( $\blacktriangle$ ), 25°C ( $\blacksquare$ ) and 37°C ( $\blacklozenge$ ); pre-immune sIgA at 4°C ( $\triangle$ ), 25°C ( $\square$ ) and 37°C ( $\lozenge$ ).



**Figure 12.12.** Release of [ $^{32}$ P]-labelled virus neutralized by sIgA and attached to BHK-21 cells by incubation with proteinase K. BHK-21 cell monolayers were inoculated with virus that had been incubated with pslgA or nsIgA for 1h at 25°C. 100  $\mu$ l of virus-antibody mixtures were incubated with the cells at the temperatures shown for 2h before being washed twice with PBS and incubated with 500  $\mu$ l proteinase K (2  $\mu$ g/ml) in PBS or 500  $\mu$ l PBS for 60 min at 4°C. The percentage [ $^{32}$ P]-labelled FPV/R released by proteinase K or by PBS was calculated relative to the total [ $^{32}$ P]-labelled FPV/R associated with cells before digestion was carried out. Each 100 $\mu$ l inoculated contained initially  $1.4 \times 10^7$  PFU, 700 HAU, and  $1.4 \times 10^5$  cpm.



internalized. The question was resolved by treating cells inoculated with virus neutralized by sIgA with proteinase K (sections 11.3; 11.3.1). This released the majority of virus attaching at 25° and 37°C (58 and 68.2% respectively), demonstrating that the majority of virus had not been internalized but remained attached to the outside of the cell. Treatment of cells that were inoculated with virus treated with pre-immune sIgA released no more than 8% cell-associated virus (Figure 12.12).

### 12.3. Mechanisms of neutralization by IgM.

The attachment of FPV/R neutralized by IgM and of non-neutralized virus to BHK-21 cells was measured as described previously. At 4°C, neutralizing IgM prevented the attachment of neutralized virus (Figure 12.13) whereas over 25% infectious virus that had been incubated with preimmune IgM had attached to BHK-21 cells after 120 min. At 25°C and 37°C similar amounts of non-neutralized virus attached as at 4°C but attachment of neutralized virus rose at 25°C to 38% and at 37°C to 47% of their respective control values after 120 minutes (Figure 12.14). This was very similar to the temperature dependence of the attachment of virus neutralized by sIgA (section 12.2). To investigate whether virus neutralized with IgM was able to undergo any of the subsequent stages of infection as it does when neutralized with IgG, the levels of virion RNA which accumulated in the nuclei were measured. Figure 12.15 shows that even though a substantial

Figure 12.13. Kinetics of attachment of non-neutralized [ $^{32}$ P]-labelled FPV/R and virus neutralized with IgM to BHK-21 cells at 4°C. Virus was first incubated for 1 h at 25°C with neutralizing IgM (●) or pre-immune IgM (○). Infectivity was neutralized (by 99%) only by the former. The subsequent procedure is described in Figure 12.1.1, except that each 100  $\mu$ l inoculated initially contained  $1.5 \times 10^7$  PFU, 630 HAU and  $1.34 \times 10^5$  cpm.

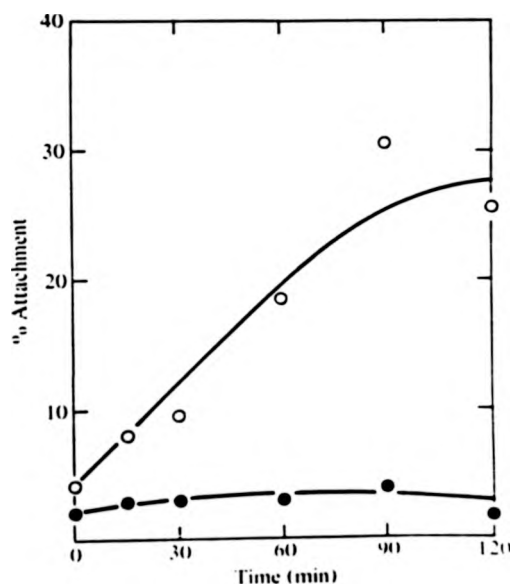


Figure 12.14. Kinetics of attachment of neutralized and non-neutralized [ $^{32}$ P]-labelled FPV/R to BHK-21 cell monolayers at 4, 25 and 37°C. Virus was first incubated for 1 h at 25° with neutralizing IgM (solid symbols) or pre-immune IgM (open symbols). The immune IgM neutralized virus by 99%. Each 100  $\mu$ l initially contained  $1.5 \times 10^7$  PFU, 631 HAU and  $1.34 \times 10^5$  cpm. Neutralizing IgM at 4°C (●), 25°C (▲) and 37°C (■); pre-immune IgM at 4°C (○), 25°C (△) and 37°C (□).

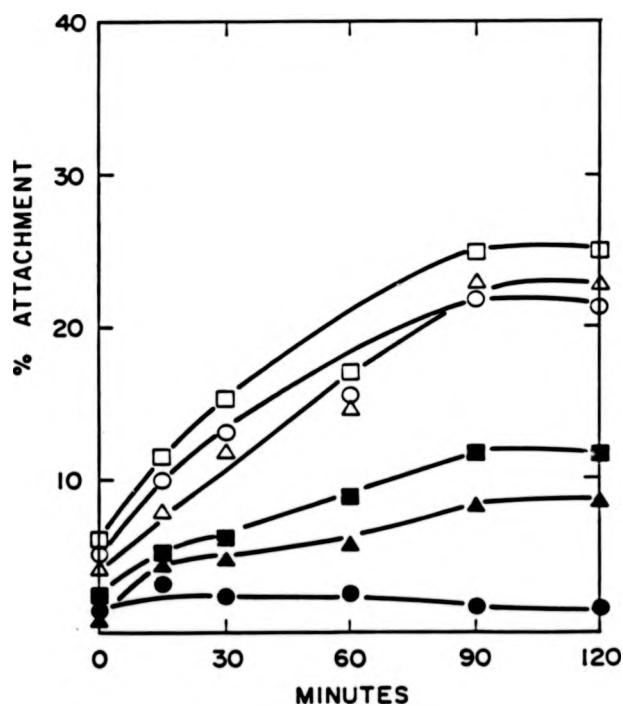
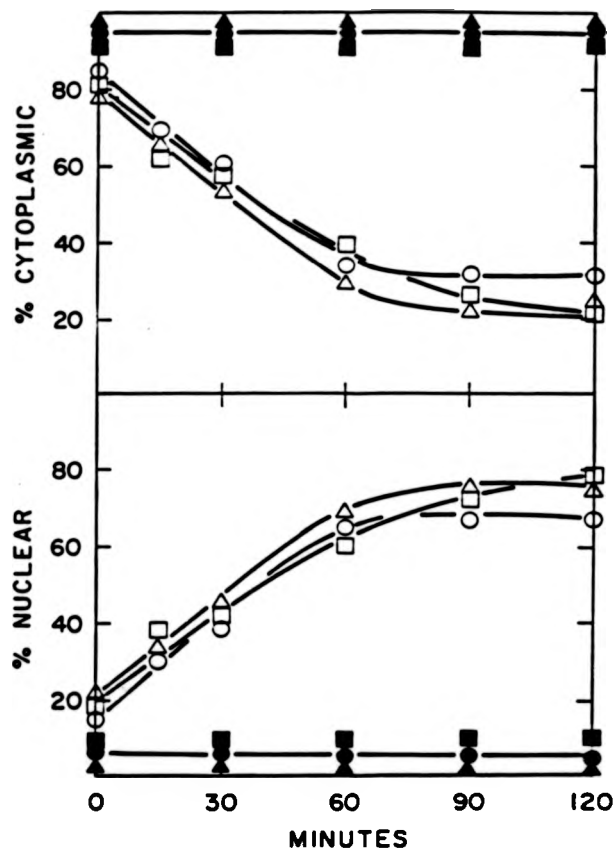
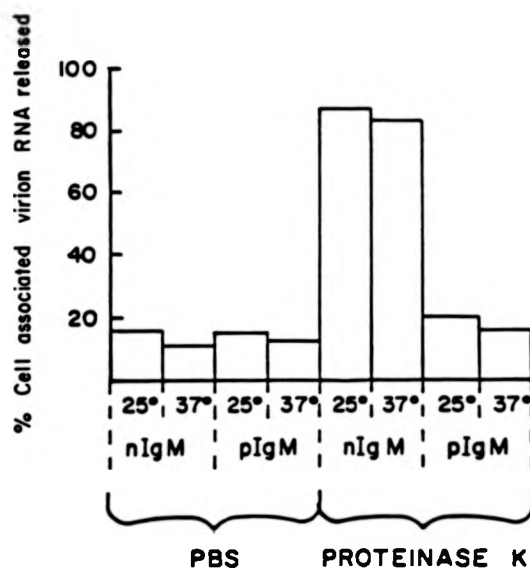


Figure 12.15. Kinetics of migration of [ $^{32}$ P]-labelled virion RNA from cytoplasm to nucleus in BHK-21 cells inoculated with virus neutralized by IgM. Virus was neutralized and inoculated at 4, 25 and 37°C as described in Figure 12.14. The immune IgM neutralized virus by 99%. Neutralizing IgM at 4°C (●), 25°C (▲) and 37°C (■); pre-immune IgM at 4°C (○), 25°C (△) and 37°C (□).



**Figure 12.16.** Release of attached  $^{32}\text{P}$ -labelled virus neutralized by IgM. Performed as described in Figure 12.14, except that virus was incubated with either neutralizing IgM or pre-immune IgM. The neutralizing IgM reduced infectivity by 99%. Each 100ul of inoculum initially contained  $1.5 \times 10^7$  PFU,  $6.31 \times 10^2$  HAU and  $1.34 \times 10^5$  cpm



proportion of virus neutralized with IgM attached to the cells at 25 and 37°C, virion RNA did not travel to the nucleus. However, as previously indicated (section 12.2) this procedure does not discriminate between virus attached to the outside of the cell and virus which has been fully internalized into the cytoplasm. To remove virus putatively attached to the outside, cells were treated with proteinase K (sections 11.3; 11.3.1). Figure 12.16 shows that 80% or more virion RNA was released from those cells which had been inoculated with virus neutralized by IgM compared to less than 20% released from cells receiving non-neutralized virus. This clearly demonstrated that the majority of attached neutralized virus is remaining on the outer surface of the cell.

Table 12.2 presents a summary of the interactions of FPV/R neutralized with different classes of immunoglobulin with BHK-21 cells at 37°C. When neutralized by either IgG or monomeric IgA, FPV/R attached to and penetrated BHK-21 cells at a rate indistinguishable from that of non-neutralized virus. In contrast the polymeric immunoglobulins sIgA and IgM blocked attachment of 50 and 47% of the neutralized virus respectively. None of the attached virus was internalized.

#### 12.4. Mechanisms of neutralization by a "manufactured" IgG dimer.

In another series of experiments virus neutralized with IgG



Table 12.2. Summary of interaction of FPV/R with BHK-21 cells after being neutralized with different classes of neutralizing immunoglobulin.

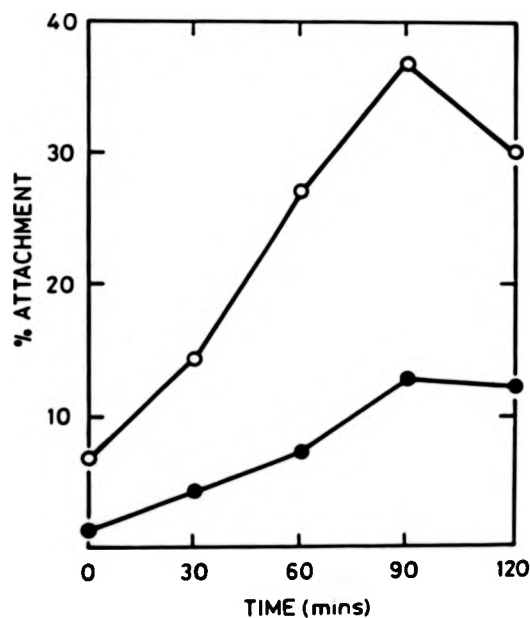
Purified antibody <sup>a</sup>	Attachment	Internal-ization	Penetration	% Neutral-ization
sIgA	++	-	-	98.5
IgM	++	-	-	99.0
IgG	++++	++++	++++	99.5
IgAb	++++	++++	++++	99.5
monoclonal IgG	++++	++++	++++	99.99

All mixtures of virus and neutralizing (n) antibody were held at 25°C for 1h before inoculation onto BHK-21 cell monolayers and each parameter was measured after 120 min incubation at 37°C. The value obtained was compared to that observed with non-neutralized virus (treated with purified antibody from pre-immune sources), +++++. At 4°C IgM and sIgA blocked attachment by about 50%. ++. Internalization was determined by evidence of penetration and by the ability of proteinase K treatment (section 11.3) to remove virus from the cells (-). Penetration is defined by the presence of virion RNA in nuclear fractions (section 11.2).

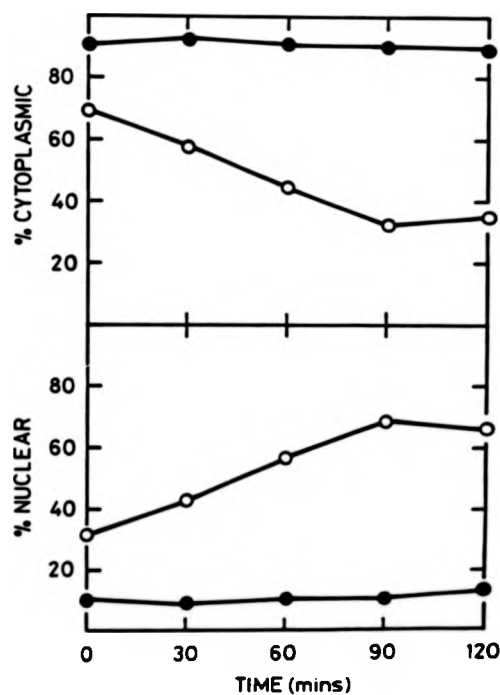
<sup>a</sup> All from polyclonal sources unless stated.

<sup>b</sup> Derived from purified sIgA by differential reduction.

Figure 12.17.1. Attachment of [ $^{32}$ P]-labelled FPV/R neutralized by a polymeric IgG complex to BHK-21 cells at 37°C. Virus was first incubated for 1h at 25°C neutralizing monoclonal IgG then incubated for a further hour with rabbit anti-mouse IgG (Fc specific; RAM) (●) or incubated with neutralizing monoclonal IgG followed by non-specific rabbit IgG (○). The neutralizing IgG plus RAM (polymeric IgG complex) reduced infectivity by 98%. Virus-antibody mixtures were then inoculated (100 ul) onto BHK-21 cells at 37 °C. The subsequent procedure is described in Figure 12.1.1, except that each 100 ul initially contained  $1.4 \times 10^7$  PFU, 700 HAU, and  $1.4 \times 10^5$  cpm.



**Figure 12.17.2.** Kinetics of migration of [ $^{32}$ P]-virion RNA from the cytoplasm to nucleus in cells inoculated as described in Figure 12.17.1. Fractionation was performed as previously described (section 11.2; Figure 11.1.2). Symbols as in Figure 12.17.1.



was reacted with rabbit anti-mouse IgG (Fc region specific) (RAM) (Nordic Immunological Laboratories) to investigate if this would cause IgG to behave like a polymeric antibody. Neutralizing mouse monoclonal IgG (HC2) was incubated with [ $^{32}$ P]-labelled FPV/R for 1h at 25°C and subsequently RAM was added for a further 1h at 25°C. Figure 12.17.1 shows the kinetics of attachment of [ $^{32}$ P]-labelled virus to BHK-21 cells at 37°C. Neutralizing IgG plus RAM reduced attachment of virus to BHK-21 cells to 40% of that of virus treated with neutralizing IgG and control rabbit IgG (purified from normal rabbit sera). At 4°C there was very little attachment. A non-specific monoclonal IgG (171/1) plus RAM had no effect on attachment. Thus neutralization by this manufactured "mock polymeric antibody" proceeded in the manner observed with sIgA (section 12.2) and IgM (section 12.3). It was further found (Figure 12.17.2) that the RNA of neutralized virus that did attach at 37°C in the above experiment remained in the cytoplasmic fraction which is consistent with the view that neutralized virus is remaining on the surface of the cells, as seen with virus neutralized by either sIgA or IgM.

#### 12.5. Mechanisms of neutralization by antibody fragments derived from monoclonal IgG

To determine if the Fc region and the bivalent character of IgG is necessary for neutralization, virus was reacted with F(ab')<sub>2</sub> and Fab' fragments (section 10.8). F(ab')<sub>2</sub> fragments neutralized FPV/R with about the same efficiency as the IgG from

which it was derived. Table 12.3 shows that neutralizing  $F(ab')_2$  fragments failed to inhibit the attachment of neutralized virus to BHK-21 cell monolayers and migration of the virion RNA to the nucleus after incubation for 90 and 120 min at 37°C. Thus neither neutralization nor attachment of neutralized virus to BHK-21 cells is dependent on the  $F_c$  region.

Neutralization by  $Fab'$  had about the same efficiency as that of IgG even though the  $Fab'$  was standardized with IgG and  $F(ab')_2$  on a molar basis which meant that the  $Fab'$  preparation had half the number of antigen binding sites (Table 12.3). In general this agrees with earlier work with polyclonal antibody (Lafferty, 1963a) and it is concluded that bivalency is not necessary for neutralization. However this particular  $Fab'$  undergoes dilution dissociation with restoration of virus infectivity (section 10.11; A.S. Carver, personal communication) which underlies the importance of antibody bivalency under normal physiological conditions. When it was necessary to employ a dilution step to measure the extent of neutralization by (i.e. after neutralization of high titre radiolabelled virus) the neutralized virus was first reacted with rabbit anti-mouse IgG ( $Fab'$  specific). This stabilized neutralization, presumably by restoring bivalency and resurrected infectivity was no longer apparent upon dilution.

Figure 12.18.1 shows that attachment of virus neutralized with  $Fab'$  was slightly reduced compared to that of virus treated with non-specific  $Fab'$  (obtained from digestion of a non-specific

Table 12.3. Percentage attachment of FPV/R and nuclear accumulation of FPV/R genomes from such virus neutralized by F(ab')<sub>2</sub> fragments to BHK-21 cell monolayers.

Time on BHK-21 cells	Attachment(%)		Accumulation in nucleus (%)	
	pF(ab') <sub>2</sub>	nF(ab') <sub>2</sub>	pF(ab') <sub>2</sub>	nF(ab') <sub>2</sub>
90 min	32	33	71	73
120 min	35	34	74	69

[<sup>32</sup>P]-labelled FPV/R was incubated for 1h at 25°C with equivalent amounts (20 ug/ml) of either neutralizing, (n) or non-neutralizing (p) F(ab')<sub>2</sub> fragments. Percentage nuclear accumulation (penetration) in parentheses. Conditions were as described in Fig. 12.18.1 and 12.18.2. Neutralizing F(ab')<sub>2</sub> fragments reduced infectivity by 98.5%.

Figure 12.18.1. Attachment of non-neutralized [ $^{32}$ P]-labelled A/FPV/Rostock/34 (H7N1) incubated with non-neutralizing Fab' (○) and virus incubated with neutralizing Fab' fragments (●) to BHK-21 cell monolayers. Virus was incubated at 25°C for 1h with equivalent amounts (8.5  $\mu$ g/ml) of Fab' fragments then 100  $\mu$ l was inoculated onto monolayers of BHK-21 cells in Petri dishes (6 x 10<sup>6</sup>/dish) at 37°C. Each inoculum contained initially 9 x 10<sup>7</sup> PFU, 200 HAU, and 1.6 x 10<sup>5</sup> cpm. The subsequent procedure is described in Figure 12.1.1. Neutralizing Fab' fragments reduced infectivity by 98%.

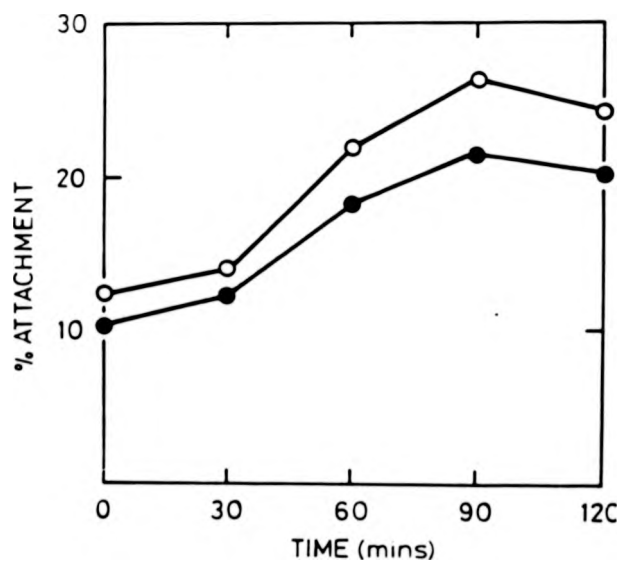
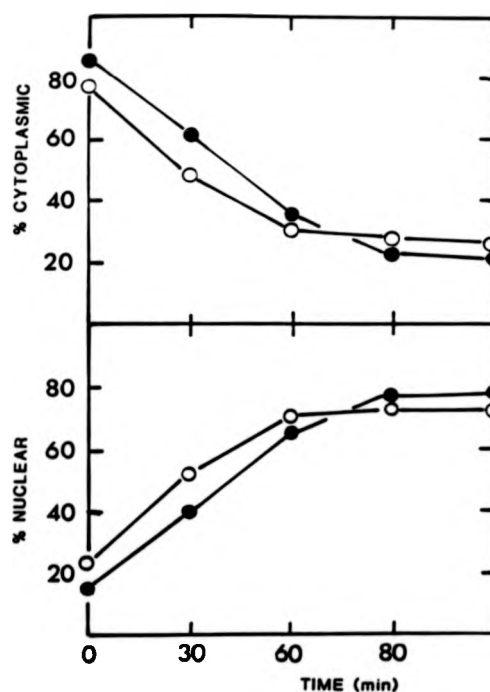


Figure 12.18.2. Kinetics of migration of [ $^{32}$ P]-virion RNA from the cytoplasm to nucleus in cells inoculated with virus neutralized by Fab' fragments (●) or virus treated with non-neutralizing Fab' fragments (○) as described in Figure 12.18.1. Fractionation was performed as previously described (section 11.2; Figure 11.1.2).





monoclonal antibody, 171/1. The nuclear accumulation of [ $^{32}$ P]-labelled RNA from FPV/R neutralized by Fab' fragments proceeded at a rate similar to that of RNA from non-neutralized virus (Figure 12.18.2). This confirmed that the penetration, and transport of the influenza virus genome to the nucleus was unimpaired by neutralizing Fab' fragments and that none of these processes in neutralized virus depends upon the Fc region or the bivalent character of IgG.

## 12.6. DISCUSSION

These studies suggest that the mechanism of neutralization of FPV/R is dependent on the class of the neutralizing immunoglobulin as represented by the different physical properties of each of these classes.

FPV/R neutralized by polyclonal IgG attached to a variety of different cell types (CEF, Ls29, HFF 4, and BHK-21 cells) with kinetics indistinguishable from those of non-neutralized virus. Further studies demonstrated that FPV/R neutralized by monoclonal IgG or polyclonal monomeric IgA (derived from sIgA by differential reduction, section 10.10.3) attached to BHK-21 cells at the same rate as non-neutralized virus. Using a fractionation technique to isolate nuclei from BHK-21 cells, it was found that neutralization of influenza virus by IgG or monomeric IgA did not affect penetration or transport of viral RNA to the nucleus. In fact, the rate of nuclear accumulation of [ $^{32}$ P]-labelled RNA from neutralized virus was indistinguishable from that of non-neutralized virus, therefore loss of infectivity must result from the inhibition of a subsequent stage of infection (Possee et al., 1982). As there was no difference in the early stages of infection between virus neutralized by IgG or by monomeric IgA it may be that IgA and IgG have the same mechanism of neutralization.

12.6.1. Why both neutralizing IgG and monomeric  
IgA fail to inhibit attachment.

The HA spike on the surface of an influenza virus is composed of three monomers each with a virus attachment site

(VAS) in a pocket surrounded by antigenic determinants to which neutralizing antibody must bind (section 2.2; Wilson et al., 1981; Rogers et al., 1983). Neutralizing IgG antibodies do not bind to the HA VAS itself. Studies with [ $^{125}$ I]-labelled IgG and intact viruses (section 13) show that at saturation only one molecule is bound per HA trimer. This could leave the VASs on the two adjacent monomers freely available for binding to CRUs and explain why neutralizing IgG or monomeric IgA does not prevent attachment. In contrast to the studies with cultured cells as described above, neutralized FPV/R does not bind to chicken erythrocytes (Possee, 1981). The influence of host cell on the neutralization of virus is well documented (section 9.1) and the binding of neutralized influenza virus to cultured cells could highlight the difference in the topography of the CRUs of erythrocytes and the CRUs on other cells (section 3.1; section 9.2.1). Glycophorin A sialoglycoproteins, the main molecules carrying CRUs for influenza viruses on erythrocytes (section 3.1; Jackson et al., 1973) are short (5 nm) molecules that lie amongst a dense web of carbohydrate and larger molecules on the erythrocyte cell surface (section 3.1; Viitala and Jarnefelt, 1985). The phenomenon of HI could therefore be a reflection of the nature of the erythrocyte surface topography. The barrier of IgG molecules on an influenza virus is successful in blocking attachment to erythrocytes. However, this "fringe" of IgG or IgA molecules may be penetrated by longer and less impeded receptors (as yet uncharacterized) on cultured cells.

Yoden et al. (1982) found that haemagglutination by influenza A/duck/Hokkaido/5/77 (H3N2) was not inhibited by neutralizing polyclonal IgG. Also Kida et al. (1982) found that some groups of monoclonal antibodies to influenza A/seal/Mass./1/80 failed to inhibit haemagglutination of intact virus yet effectively neutralized virus infectivity. The physical presence of the IgG molecules in these examples cannot be sufficient to prevent CRU-VAS interaction, a reflection of the situation with BHK-21 cells and other cultured cells, suggesting that neutralization is at a later step than adsorption.

Burton (1986), challenges the traditional view that IgG molecules bound to antigen remain in a undistorted Y-shape. He proposes that IgG molecules in interaction with antigen can be dislocated so that the Fc portion of the molecule moves with respect to the two Fab arms bound to the antigen. This distortion also occurs with IgM and is important in the interaction of antibody with complement and Fc receptors. The occurrence of an IgG or IgA in a dislocated Y-shape rather than the conventional Y-shape or T-shape (section 7.1) may be a crucial feature in the interaction of antibody-virus complexes with CRUs. The distortions of IgG or IgA molecules could result in an inconsistent barrier allowing CRUs on BHK-21 cells and other cultured cells to gain access to VASs on the HA spikes.

#### 12.6.2. How does IgG neutralize FPV/R?

Possee et al. (1982) showed that FPV/R neutralized by IgG

has reduced transcriptase activity both *in vivo* and *in vitro*. There is considerable evidence that antibodies can mediate conformational changes in antigens and that these may be transmitted through and along molecules (section 9.2.2). Since the transcriptase function, situated internally in the virus, is modified by antibody on the other side of the viral envelope it has been proposed that the effect is mediated by conformational change. The binding of neutralizing immunoglobulin could trigger changes in the tertiary structure of the HA which are transmitted across the envelope via the transmembrane segment of HA2 to the transcriptase.

However, the reduction in transcriptase activity by neutralizing IgG is only three to ten fold; which does not correlate with the neutralization titre of 99.99% (Possee et al., 1982). Recent work (R.Rigg, unpublished data) has shown that neutralizing monoclonal IgG has little effect on the *in vitro* messenger RNA primed transcription of FPV/R RNA. Further investigation of the molecular mechanism of influenza neutralization (R.Rigg, unpublished data) suggests that a secondary uncoating step (Bukrinskaya et al., 1982) could be inhibited by neutralizing antibody and that this results in the delivery of non-infectious sub-viral particles to the nucleus. It may become apparent that an accumulative affect of neutralizing antibody on these post attachment phases (uncoating and transcription) is responsible for loss of infectivity.

Competitive binding radioimmunoassays on isolated influenza virus HA have demonstrated that binding of certain monoclonal antibodies increases the binding of other monoclonal antibodies to different epitopes due to conformational changes within the HA (Lubeck and Gerhard, 1982). Studies with [ $^{125}\text{I}$ ]-labelled neutralizing IgG and intact influenza virus have shown that at saturation only one IgG molecule is bound per HA spike (section 13). This prevents the further binding of not only monoclonal antibodies to different epitopes but polyclonal IgG (section 13.5) and polyclonal sIgA (section 13.7.1). These results mean that studies indirectly demonstrating conformational change on FPV/R HA by neutralizing antibody by its effect on subsequent antibody binding on intact FPV/R particles would be unlikely to succeed. Susceptibility of HA to proteolytic enzymes provided evidence that HA had undergone a conformational change at low pH (Skehel et al., 1982). However, studies with a range of proteolytic enzymes (trypsin, chymotrypsin, subtilisin, and elastase) on neutralized and non-neutralized virus failed to demonstrate any evidence of differing susceptibility to enzyme digestion (G. Ward and N.J. Dimmock, unpublished data). The physical presence of the neutralizing IgG bound to the HA may inhibit the action of enzymes, therefore preventing this technique from being definitive. Chemical cross-linking studies failed to detect conformational changes in the HA1, HA2, NP or M proteins of neutralized FPV/R. Circular dichromism studies of neutralizing IgG-virus complexes could prove as useful to the study of influenza virus neutralization as they did in studies of

pH induced conformational changes in BrHA (Skehel et al., 1982). Small angle X-ray or neutron scattering investigations (Review: Cusack, 1982) could be used to investigate the effect of neutralizing immunoglobulins on gross influenza virus structure. The scans obtained from small angle X-ray scattering studies of neutralized influenza virus would not only provide insight into the possibility of conformational changes in the HA spikes, but also within the virus particle itself.

One problem of the studies with neutralizing immunoglobulin is in estimating the extent to which aggregation contributes to neutralization. However, it is known that under similar experimental conditions used in this thesis, virus neutralized with IgG has the same sedimentation velocity as non-neutralized virus (A.S. Carver and N.J. Dimmock, unpublished data). This indicates that aggregates, if there are any, are not large. Electron microscope studies on neutralization by IgG and polymerization (aggregation) also indicated that at high IgG concentration to virus particle ratios similar to those used in my studies there is minimal aggregation (S.J. Armstrong and N.J. Dimmock, unpublished observations). In fact most (> 95%) virus particles are monomeric and are completely coated with IgG. Aggregation only occurs at lower IgG to virus ratios. High concentrations of sIgA also resulted in virus monomers coated with sIgA. However, IgM caused significant polymerization in conditions similar to my studies.

Both  $F(ab')_2$  and  $Fab'$  neutralized virus efficiently indicating that neither the  $Fc$  region or bivalency respectively are necessary prerequisites. Virus neutralized by  $Fab'$  attached to cells and was internalized, showing that neither the  $Fc$  region or bivalency is responsible for the behaviour of neutralized virus (Lafferty 1963b). However experience with poliovirus suggests that under some circumstances  $Fab'$  fragments from certain monoclonal IgGs are not neutralizing (section 9.2.2) so no generalization is yet possible.

Interestingly the amount of virus neutralized by  $Fab'$  which attached to the BHK-21 cells was consistently slightly lower than that of non-neutralized virus or virus neutralized by IgG. It may be that because of its smaller size, more  $Fab'$  can bind per HA spike than IgG and thus impede attachment by steric hindrance.

#### 12.6.3. Neutralization by sIgA and IgM.

Influenza virus neutralized by sIgA and IgM behaved in the same way, perhaps indicating a common mechanism of neutralization. At 4°C neutralizing sIgA and IgM effectively prevented attachment of influenza. Steric hindrance of the attachment of virus neutralized by IgM and sIgA is probably the mechanism of neutralization at 4°C but it is possible that virus has already been rendered non-infectious by the same mechanism that operates with IgG and sIgA. The extent to which aggregation by these antibodies contribute to neutralization has yet to be



determined. At 25 and 37°C half the virus neutralized by either IgM or sIgA attaches to cells presumably through increased thermal agitation bringing VASs and CRUs into the correct juxtaposition. Very little of this attached virus was internalized and it is apparent that IgM and sIgA interfere with the endocytotic event responsible for internalization. It is not known why such virus is not endocytosed into vesicles (section 3.2) but the physical presence of these polymeric immunoglobulins may jam the circumferential adherence or "zipper" mechanism of endocytosis (Griffin et al., 1975; Marsh, 1984). The curtailment of endocytosis by neutralizing sIgA and IgM at 25 and 37°C is so substantial that the majority of neutralized virus particles can be removed from the surface of BHK-21 cells by proteinase K treatment. Virus neutralized by an IgG + anti-(IgG Fc) complex interacted with cells in the same manner as virus neutralized with sIgA or IgM supporting the idea that the physical size of a polymeric antibody, unlike IgG and IgA, impedes attachment (especially at 4°C) and prevents successful endocytosis. The effective agglutination characteristics associated with the pentameric and dimeric structures of IgM and sIgA respectively could also be relevant.

Attachment to cells without internalization at physiological temperatures by neutralizing IgM may be relevant *in vivo*, it could lead to complement-mediated cell lysis early in the disease (section 5.4; Lachmann, 1985). sIgA does not fix complement (Tomasi, 1976) but can mediate ADCC (section 5.4; Tagliabue et

al., 1983), therefore the presence of sIgA-virus complexes on cells *in vivo* could be important in the elimination of infection. Neutralizing sIgA obtained from the milk of pigs did not inhibit the attachment of transmissible gastroenteritis virus (TGEV) to porcine derived cell lines (Nguyen et al., 1986). However, the neutralized TGEV was unable to enter cells as demonstrated by the action of proteinase K which removed it from the cell surface. These are similar results to those reported in this thesis for the neutralization of influenza virus by sIgA at 25 and 37°C.

#### 12.6.4. In what way could IgM and sIgA physically block attachment?

Since IgM is pentameric (i.e. composed of 5 four chain immunoglobulin units; section 6.2.2) as well as binding in a spider-like conformation (section 7.1; Figure 7.1.3) with all Fab' $\mu$  arms to the influenza HAs, it is possible that it could bind with only a few Fab' $\mu$  with the rest of the molecule providing a physical wedge between virus and CRUs (Figure 7.1.3). Since sIgA is composed of 2 four chain immunoglobulin units linked by the Sc and J chain (section 6.2.3), sIgA may bind in a "hair-pin" conformation (evidence suggests that sIgA is flexible enough to have this conformation; section 7.1; Figure 7.1.3), with both four chain units attached to the virus, or through only one unit so that the sIgA molecule would be orientated in the double Y extended conformation perpendicular to the virus surface (Figure 7.1.3). This latter docking orientation would

approximately double the distance between VAS and CRU achieved when IgG or monomeric IgA binds. This measure of steric hindrance may be crucial and could account for the failure of IgG and monomeric IgA to prevent attachment (see above).

The determination of the mechanism of neutralization of influenza virus by sIgA is of particular interest, since in the respiratory tract of man neutralizing sIgA is a major determinant of resistance to re-infection with influenza virus (section 5.5). For example, local sIgA memory for influenza HA has been demonstrated in the upper respiratory tract of humans challenged with an inactivated vaccine (Wright et al., 1983). IgG and IgM can also contribute to human local immunity but are present in low concentrations compared to that of sIgA. The problem of obtaining sufficient amounts of immune sIgA for the studies in this thesis was overcome by using bile from appropriately immunized rats (section 10.10). This method could be adapted to investigate neutralization by sIgA of other viruses. sIgA comprises approximately 70% of the total immunoglobulin in bile (Andrew and Hall, 1982) and immunochemical analysis has established (Andrew and Hall, 1982; Tomasi and Bienenstock, 1968; Nagura et al., 1981) that sIgA from bile and respiratory secretions is identical. Stimulation of the gastrointestinal tract of human volunteers established neutralizing sIgA in the respiratory secretions (Waldman et al., 1981). Monomeric IgA is present in the serum of mammals, comprising 20% of normal serum immunoglobulins in man (Nisonoff et al., 1975; Tomasi, 1976), but

definitive studies of its role in resistance to influenza infections have not been carried out. Systemically the neutralization of influenza virus is a function primarily of IgG (section 5.5).

In conclusion, the mechanism of neutralization of influenza virus A/fowl plague virus/Rostock/34 (H7N1) is clearly dependent on the class of neutralizing immunoglobulin. Only at 4°C does neutralizing sIgA and IgM effectively prevent attachment of influenza virus. At physiological temperatures IgM and sIgA permit the attachment of approximately half the neutralized virus but this is not internalized. In contrast, neither neutralizing IgG or IgA has any effect on attachment at temperatures of 4 to 37°C, or on the subsequent stages of infection up to the accumulation of virion RNA in the nucleus. Functional Fab fragments from monoclonal IgG reduced only slightly the penetration of neutralized FPV/R to BHK-21 cells, whereas FPV/R neutralized by F(ab')<sub>2</sub> fragments attached and penetrated BHK-21 cells to the same extent as infectious FPV/R.

It is emerging that virus neutralization is a tripartite system inter-dependent on cell, virus and immunoglobulin. Studies on the mechanisms of influenza neutralization in the natural target tissues of the virus in the mammalian host (i.e. those of the upper respiratory tract) using homologous immunoglobulin need to be done to yield information on the relevant mechanisms which operate *in vivo*.

**SECTION 13:**

**THE NUMBER OF IMMUNOGLOBULIN MOLECULES BINDING TO FPV/R  
HAEMAGGLUTININ ON INTACT FPV/R PARTICLES.**

### Summary

Radiochemical data in this investigation on the binding of immunoglobulins to the HA of FPV/R in suspension suggest that the virus is saturated at approximately one IgG molecule per HA spike. sIgA saturates at a level of 0.4 (the equivalent of 0.8 four chain immunoglobulin units) per spike and IgM at a level of 0.1 (the equivalent of 0.5 four chain units) per spike. Two-step competition assays show that the binding of one molecule of monoclonal IgG to a HA spike prevented the binding of monoclonal antibodies to two other epitopes on that spike and also the binding of polyclonal antibody to the HA.

Kinetics of neutralization of FPV/R with monoclonal IgG under all conditions, except low temperature or minimal neutralizing concentrations of antibody, were single-hit. However at 4°C with a low concentration of antibody kinetics were multi-hit and extrapolation suggested that about 3 IgG molecules were required to neutralize an infectious virus particle. This is at variance with radiochemical data showing that at least 50 antibodies per virus particle are required for neutralization. The apparent conflict can be reconciled by a theory that neutralization only occurs when antibody binds to certain "neutralization relevant" HA spikes which are in the minority. Binding to the majority of HA spikes is irrelevant to neutralization. The neutralization relevant and irrelevant spikes would differ only in their interaction with the virion core structure.

### Introduction

Studies on the mechanisms of neutralization of FPV/R (section 12; Possee 1981; Possee et al., 1981) have demonstrated that FPV/R neutralized to greater than 99.9% by polyclonal or monoclonal IgG attaches to mammalian and avian cells at the same rate as non-neutralized virus. In order to understand the quantitative aspects of the neutralization reaction more fully an estimate of the number of IgG molecules binding to FPV/R particles is required.

Kinetic data (section 8.1.1) show that neutralization follows either single-hit or multi-hit kinetics with at most 3 hits required to inactivate one infectious virus particle. The aim of this investigation was to obtain direct biochemical evidence, using radiolabelled antibody, of the minimum number of antibody molecules per particle which are required to achieve neutralization and to correlate this with kinetic data.

The kinetic data also suggest that as one or a few neutralizing IgG molecules are bound to the surface of an influenza virus particle the vast majority of potential VASs would be free of antibody. If this is the case, then one would expect the unencumbered HA to be able to attach virus to a cell. Firstly, to examine this problem and to address in part how neutralized virus attaches to cells (section 12), the number of HA spikes on an influenza particle was determined. Then, using radiolabelled antibody molecules, the maximum number of immunoglobulin molecules binding to each HA spike was calculated.

It was determined that one bound immunoglobulin molecule had the ability to interfere with the binding of antibodies to vacant identical, or even different epitopes, on the same HA spike.



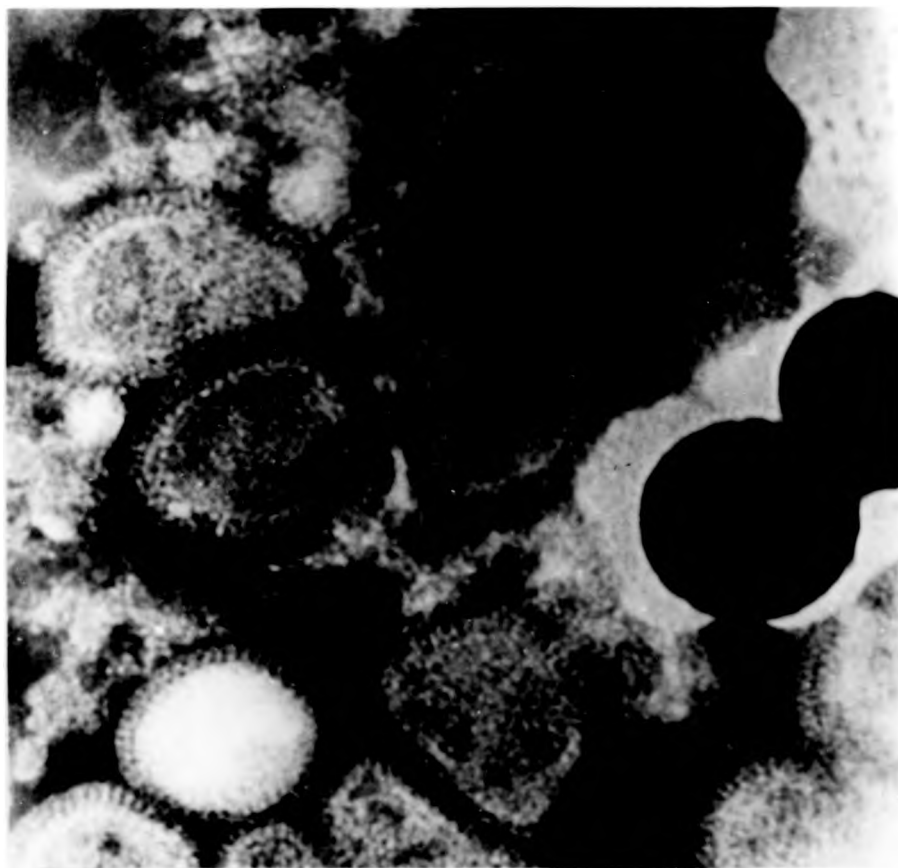
### 13.1. Estimation of the number of surface projections.

The number of surface projections was estimated using the method of Tiffany and Blough (1970b). The mean overall diameter ( $D_o$ ) of a FPV/R particle was 119.5nm ( $\pm 23.2$ ;  $n=100$ ) (Figure 13.0.1). The diameter of non-spherical (quasispherical) particles was estimated as an average of three axes; the longest axis, the shortest axis and an intermediate axis. The diameter of the virus particle at the enveloped surface ( $D_e$ ) was calculated by  $119.5\text{nm} - 2 \times \text{external length of a spike (24 nm)}$ ;  $D_e = 95.5\text{nm}$ . The mean area of a FPV/R particle ( $D_e^2 \pi$ ) was 28652 nm<sup>2</sup>. The spacing between the spikes was 5.1 nm ( $\pm 1.9$ ;  $n=50$ ) (Figures 13.0.2 to 13.0.5). For spikes spaced that distance ( $a$ ) apart, the mean area per spike surrounded by six others is  $0.5 \times a^2 \sqrt{3}$  which for FPV/R was found to be 22.52 nm ( $\pm 2.08$ ). The mean number of spikes per particle was found to be 1273 spikes ( $2 D_e^2 \pi / a^2 \sqrt{3}$ ).

#### 13.1.1. Estimation of the number of virus particles per HAU FPV/R.

The number of FPV/R particles per HAU was determined by diluting latex spheres of pre-determined concentration with a known HAU of FPV/R. Using an electron microscope each particle was scored. The latex particles were scored for the initial dilution and the conversion factor used is also the factor required to convert the FPV/R count to total particles. In this manner ten separate determinations were made. The mean value was

**Figure 13.0.1. Electronmicrograph of influenza virus particles visualized by negative stain with 1% sodium silicotungstate. 109 nm (SD  $\pm$  2.7 nm) diameter latex beads (B) were used to calibrate size. The mean overall diameter ( $D_o$ ) of a FPV/R particle was 119.5 nm (SD  $\pm$  23.2; n= 100). Virus particles were approximately spherical and closely covered with projecting haemagglutinin and neuraminidase spikes. Some can be seen end on and around the periphery of the particle (x 312,000).**



**Figure 13.0.1. Electronmicrograph of influenza virus particles visualized by negative stain with 1% sodium silicotungstate. 109 nm (SD  $\pm$  2.7 nm) diameter latex beads (B) were used to calibrate size. The mean overall diameter ( $D_o$ ) of a FPV/R particle was 119.5 nm (SD  $\pm$  23.2; n= 100). Virus particles were approximately spherical and closely covered with projecting haemagglutinin and neuraminidase spikes. Some can be seen end on and around the periphery of the particle (x 312,000).**

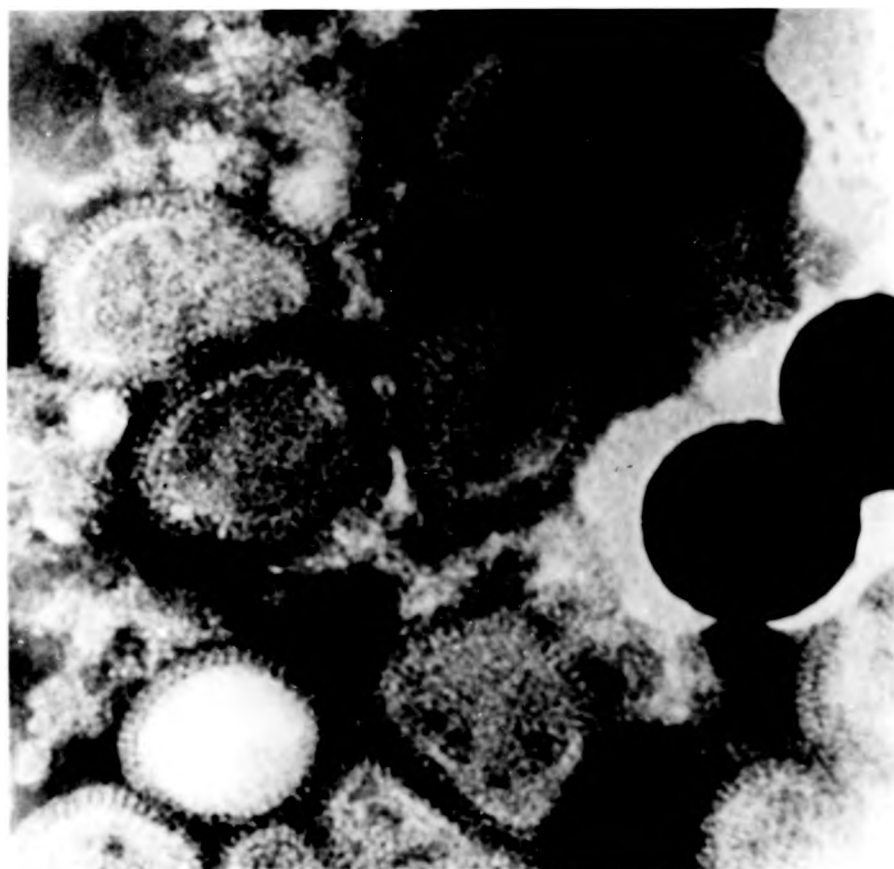


Figure 13.0.2 to 13.0.5. Negatively stained preparations of FPV/R particles. The surface spike distribution when sufficiently distinct (circled regions) permitted calculation of a mean spike spacing of 5.1 nm (SD  $\pm$  1.9; 50 measurements from 20 different particles).

Figure 13.0.2  
( $\times$  360,000)

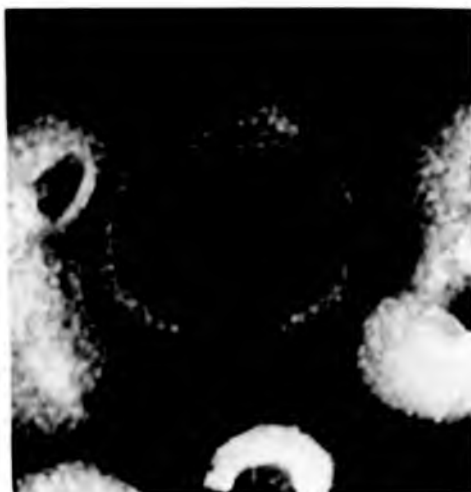


Figure 13.0.3  
( $\times$  560,000)



Figure 13.0.2 to 13.0.5. Negatively stained preparations of FPV/R particles. The surface spike distribution when sufficiently distinct (circled regions) permitted calculation of a mean spike spacing of 5.1 nm (SD  $\pm$  1.9; 50 measurements from 20 different particles).

Figure 13.0.2  
( $\times$  360,000)



Figure 13.0.3  
( $\times$  560,000)



Figure 13.0.2 to 13.0.5. Negatively stained preparations of FPV/R particles. The surface spike distribution when sufficiently distinct (circled regions) permitted calculation of a mean spike spacing of 5.1 nm (SD  $\pm$  1.9; 50 measurements from 20 different particles).

Figure 13.0.2  
( $\times$  360,000)

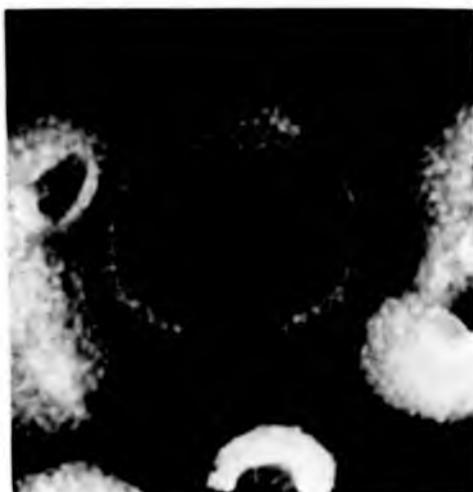


Figure 13.0.3  
( $\times$  560,000)



Figure 13.0.2 to 13.0.5. Negatively stained preparations of FPV/R particles. The surface spike distribution when sufficiently distinct (circled regions) permitted calculation of a mean spike spacing of 5.1 nm (SD  $\pm$  1.9; 50 measurements from 20 different particles).

Figure 13.0.2  
(x 360,000)



Figure 13.0.3  
(x 560,000)



Figure 13.0.4  
(x 350,000)

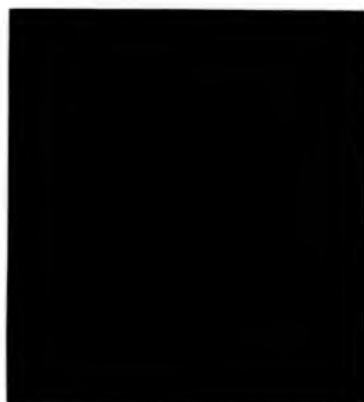


Figure 13.0.5  
(x 480,000)

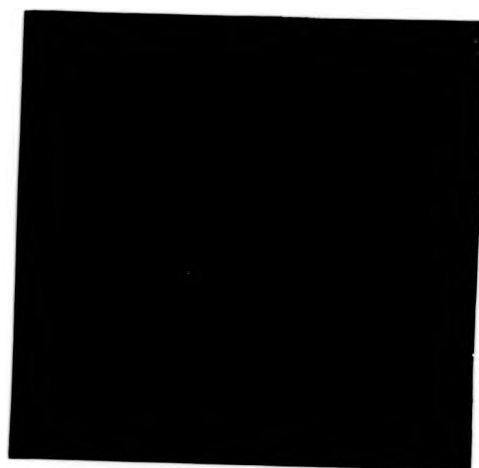




Figure 13.0.4  
(x 350,000)

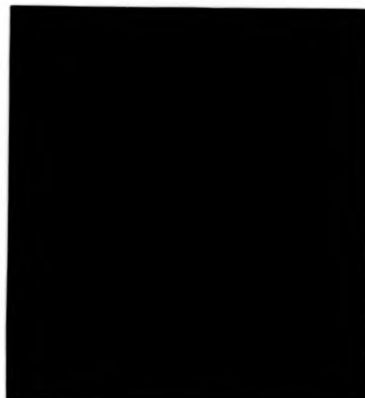
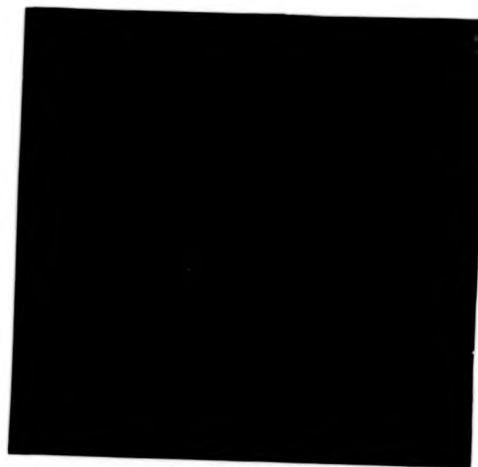


Figure 13.0.5  
(x 480,000)



1 HAU =  $3.981 \times 10^7$  particles (SD  $\pm 2.42 \times 10^6$ ).

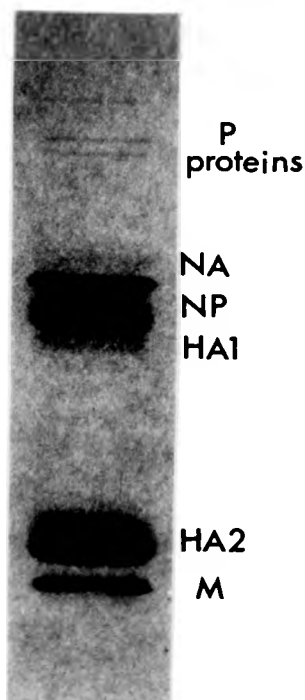
### 13.2. Estimation of the proportion of haemagglutinin (HA) and neuraminidase (NA) spikes.

Coomassie blue BSA-complexes on polyacrylamide gels gave linear plots of area under peak against  $\mu\text{g}$  protein and were shown to follow Beers law. This demonstrated that as well as being a suitable method for studying relative concentrations of polypeptides in a mixture this method is satisfactory for quantifying protein concentration (Fazekas de St. Groth et al., 1963; Skehel and Schild, 1971). By scanning the polypeptide bands stained by Coomassie brilliant blue (Figure 13.0.6) on a polyacrylamide gel with a Joyce-Loebl densitometer and measuring the areas ( $\text{mm}^2$ ) under the peaks of the scan (Figure 13.0.7) it was estimated that the ratio of HA to NA was 4.6/1 (mean of 5 measurements). This would suggest that out of an estimated 1273 spikes approximately 1000 were haemagglutinin.

#### 13.3.1. HI antibody resistant patterns defined 3 HI epitopes

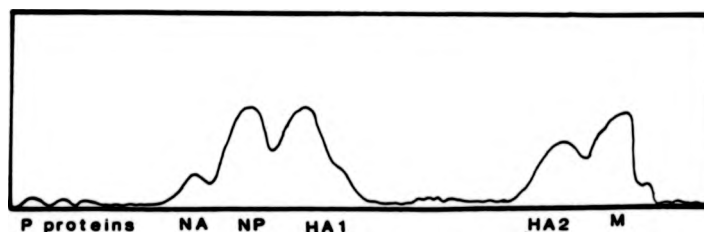
Information on the antigenic properties of monoclonal antibodies was required in order to plan and interpret investigations concerning the quantification of binding of competing monoclonal IgGs.

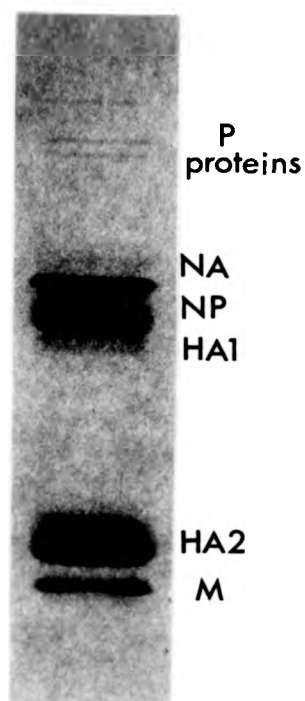
An antigenic site of a protein is defined here as a region



**Figure 13.0.6.** The polypeptide components of influenza A/FPV/R. 10,000 HAU of purified FPV/R were loaded onto and separated by SDS-PAGE as described in section 10.6. The protein bands were stained by Coomassie Brilliant Blue and then scanned in a Joyce-Loebl densitometer.

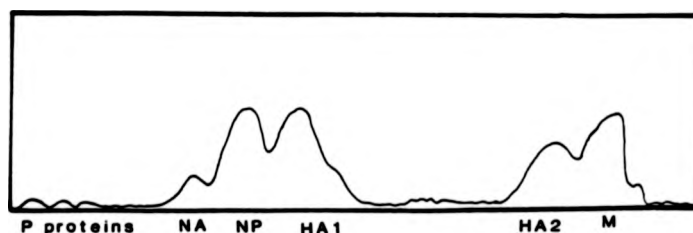
**Figure 13.0.7.** Densitometer tracing of the stained polypeptide components of FPV/R separated on an SDS-PAGE gel (Figure 13.0.6). Gels were scanned at 620 nm in a Joyce-Loebl densitometer. The percentage of total stain bound for NA and HA (HA1 + HA2) protein was calculated and from the results of 5 measurements the average HA:NA ratio was taken to be 4.6:1 (SD  $\pm$  0.6).





**Figure 13.0.6.** The polypeptide components of influenza A/FPV/R. 10,000 HAU of purified FPV/R were loaded onto and separated by SDS-PAGE as described in section 10.6. The protein bands were stained by Coomassie Brilliant Blue and then scanned in a Joyce-Loebl densitometer.

**Figure 13.0.7.** Densitometer tracing of the stained polypeptide components of FPV/R separated on an SDS-PAGE gel (Figure 13.0.6). Gels were scanned at 620 nm in a Joyce-Loebl densitometer. The percentage of total stain bound for NA and HA (HA1 + HA2) protein was calculated and from the results of 5 measurements the average HA:NA ratio was taken to be 4.6:1 (SD  $\pm$  0.6).



composed of contiguous (or non-contiguous) amino acids that induces and binds antibodies. An epitope is the molecular conformation of a sequence of amino acids (contiguous or non-contiguous) on the antigenic site that a monoclonal antibody will recognize and bind to. Therefore a single antigenic site may be capable of expressing many epitopes. The epitopes within an antigenic site may or may not overlap functionally, that is an escape mutant resistant to the action of one monoclonal antibody may or may not escape the action (i.e. HI) by other monoclonal antibodies belonging to the same cluster.

Each monoclonal immunoglobulin in mouse ascites fluid was used to select escape mutants resistant to HI by that antibody. Of 21 mutants, 12 behaved reproducibly in HI assays. Unstable mutants were not studied further. Each one of the 12 escape mutants was classified by its sensitivity to HI by each of the monoclonal IgGs. This defined a series of epitopes. The escape mutants resistant to HC61 defined epitope X; those resistant to HC10 defined epitope Y, and those resistant to HC58 and HC2 defined epitope Z (Table 13.1).

#### 13.3.2. Selection of escape mutants by polyclonal sera.

Sera WR45, WR6 (both containing IgG to FPV/D HA; H7) and WR69 (serum containing IgG to FPV/R HA; H7) were used to select escape mutants from parental virus stocks. However it was found that only WR45 could be used to select such mutants. The ability

Table 13.1. HI analysis of influenza A/FPV/R antigenic mutant viruses selected by anti-H7 monoclonal antibody.

Virus	Monoclonal IgG			
	HC61	HC10	HC58	HC2
	[X]	[Y]	[Z]	[Z]
Mutant 61B	-	+++	+++	+++
" 61D	-	++	+++	+++
" 10B	+++	-	+++	++
" 10C	+++	-	+++	+++
" 10E	++	-	+++	+++
" 10F	+++	-	+++	+++
" 58A	+++	+++	-	-
" 58C	+++	+++	-	-
" 58D	+++	+++	-	-
" 2C	+++	+++	-	-
" 2G	+++	+++	-	-
" 2H	+++	+++	-	-
FPV/R(p)	+++	+++	+++	+++
FPV/R(a)	+++	+++	+++	+++

Monoclonal antibodies HC2, HC10, HC58 and HC61 were used to select escape mutants from parental stocks (section 10.14). Epitope in parentheses.

+++ > 3.3 log<sub>10</sub> HI\*  
 ++ > 2.2 log<sub>10</sub> , < 3.3 log<sub>10</sub> HI.  
 - < 1.45 log<sub>10</sub> HI.

FPV/R(p) = parental FPV/R  
 FPV/R(c) = control passage FPV/R

\* HI titre expressed as log<sub>10</sub> reciprocal of dilution causing 50% inhibition of 4 HAU FPV/R.

n.b. Additional anti-H7 ascites fluids HC1, HC3 and M1 (gifts from J.J. Skehel) were also mapped in this study. HC1 mapped to antigenic site Z, and HC3 and M1 mapped to X. These monoclonal antibodies were not used in any other experiments.

Table 13.2. Antigenic relationship between escape mutant viruses selected by anti-H7 polyclonal antibody (WR45) and escape mutant viruses selected by monoclonal antibody as measured by HI.

Virus		Mono- or poly- clonal IgG					
		WR45	HC61	HC10	HC58	HC2	WR69
			[X]	[Y]	[Z]	[Z]	
Mutant	R45A	2.8	4.7	-	2.7	4.7	3.4
"	R45B	2.8	4.7	1.9	3.1	4.7	3.4
"	R45C	2.5	4.7	-	2.9	4.7	3.6
"	R45D	2.8	4.7	1.6	3.0	4.7	3.1
"	61B	3.4	-	4.4	2.8	NT	3.4
"	10C	3.1	4.7	-	2.8	NT	3.6
"	58D	3.6	4.7	4.4	-	NT	3.6
FPV/R(a)		3.4	4.7	4.1	3.1	4.7	3.4
FPV/R(b)		3.4	4.4	4.4	3.8	4.1	3.4
FPV/R(p)		3.4	4.7	4.4	3.8	4.7	3.4

Polyclonal WR45 was used to select escape mutants from parental FPV/R (p) (section 10.14). Escape mutants selected by WR45 are mutants R45A-D. Designation of antibodies as text.

HI titre expressed as the  $\log_{10}$  reciprocal of the dilution inhibiting haemagglutination (section 10.12). All are corrected for homologous interaction.

- < 1.45  $\log_{10}$

FPV/R (a) and (b) are control FPV/R passages.

NT = not tested.

Table 13.3. Neutralization assays as determined by plaque reduction of mutant viruses by polyclonal and monoclonal IgG.

Virus	% Neutralization * by poly- or mono- clonal IgG				
	WR45	HC61	HC10	HC58	HC2
		[X]	[Y]	[Z]	[Z]
Mutant R45A	99.99	>99.99	-	86.0	>99.99
" R45B	99.98	99.98	-	90.0	>99.99
" 61E (X)	99.99	-	>99.99	98.2	>99.99
" 10C (Y)	99.98	99.95	-	98.0	>99.99
" 58D (Z)	99.99	99.93	>99.99	-	-
" 2C (Z)	99.96	99.99	>99.99	-	-
FPV/R(a)	99.99	99.96	>99.99	98.2	>99.99
FPV/R(p)	99.99	>99.99	>99.99	98.0	>99.99

\* Neutralization of  $5 \times 10^7$  PFU.  
- < 25% neutralization



of the mutants to react with antibody was assayed by HI (Table 13.2). The escape mutant viruses (R45 A-D) selected by WR45 consistently failed to react with ascites fluid containing HC10, but essentially gave a complete pattern reactivity against the other monoclonal antibodies. An exception was the slightly lowered HI reactivity of HC58 towards the escape mutants. This is surprising since HC10 and HC58 map to different antigenic sites (Y and Z respectively) and since reactivity to HC2 (Z) was unaffected. The mutants also showed a lowered HI reactivity with WR45. WR45 significantly inhibited the haemagglutination activity of mutant 10C, a variant resistant to HC10. The reactivity of the R45 escape mutants against another polyclonal IgG, WR69, was not diminished.

The escape mutants selected by WR45 on three separate occasions were re-passaged in the presence of WR45 antibody or HC10 antibody and each remained mutant in character. No further selection occurred when these escape mutants were incubated with polyclonal sera WR6 and WR69.

An HI assay with FPV/D revealed that all monoclonal and polyclonal IgG sources reacted precisely as they did with FPV/R. This demonstrated exact serological relatedness between the FPV/D H7 and FPV/R H7, with respect to the reagents used.

Disparity between HI tests and neutralization assays have been described (Lu et al., 1982; Kida et al., 1982), therefore,

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neutralization assays were carried out on escape mutants (Table 13.3). This study revealed that the slightly reduced HI activity of WR45 towards escape mutants RasA and B was not reflected by a comparable resistance of the mutants to neutralization by WR45. The neutralization of the mutants by WR45 was, in fact, slightly more efficient than the neutralization of control passage FPV/R(a) and parental FPV/R(p). Apart from this deviation the results observed in the HI assay were reflected in the neutralization tests. WR45 neutralized the escape mutants selected by monoclonal antibodies, including the mutant selected by HC10. HC10 failed to neutralize the Ras escape mutants reflecting its inability to inhibit haemagglutination. HC58 showed a lowered capability to neutralize Ras A/B that mirrored the reduced HI activity of HC58 against these escape mutants. The correlation between the neutralization of escape mutants selected by the monoclonal immunoglobulins and the HI pattern was strong.

The results suggest that WR45 can indeed select variants, but that this capacity is limited to mutations in one epitope, Y, the epitope also recognized by HC10. However WR45 is a polyclonal serum as it retains the ability to neutralize and inhibit the haemagglutination ability of the mutant viruses it has selected. It must contain populations of IgG that bind to other epitopes apart from epitope Y. These undefined populations of IgG ensure complete neutralization of site Y mutants as further variants do not arise upon re-incubation of escape mutants with WR45.

These observations of the properties of WR45 IgG became important in investigations concerning the inhibition of binding of antibody to virus by pre-bound antibody.

The levels of neutralization of FPV/R achieved with polyclonal sIgA or polyclonal IgM although significant were not high enough to select escape mutants.

#### 13.4.1. Estimation of the number of radiolabelled polyclonal IgG molecules binding to FPV/R particles.

Table 13.4 shows that when FPV/R and purified polyclonal IgG were mixed together in a ratio giving 99.95% neutralization of the virus there were from 0.41 to 0.69 (mean = 0.53) IgG molecules binding to each haemagglutinin spike (see sample calculation, Table 13.4). The reproducibility of these results encouraged further investigation. When the ratio of IgG in terms of HIU to HAU of virus was varied (Figure 13.1.1) it was found that the maximum amount of IgG that could bind to each haemagglutinin spike was approximately 0.9 (i.e. in the order of 1). At this level neutralization was in excess of 99.5%. This pattern was similar with neutralizing IgG obtained from two different rabbits, WR26 and WR45, which had been inoculated (section 10.7) with FPV/D (H7N7) and one rabbit WR69 that had been inoculated with V31 which has an H7 derived from FPV/R

Table 13.4. Estimation of the amount of specific polyclonal IgG binding to each HA spike on an intact FPV/R particle at an HIU/HAU ratio of 3.75:1\*.

IgG designation	cpm due to specific binding	No. of IgG molecules/HA spike
WR45	8452	0.56
"	8960	0.58
"	10559	0.69
"	6298	0.41
WR26	10521	0.42

\* 20 HAU FPV/R ( $7.962 \times 10^8$  virus particles) were incubated with 75 HIU purified IgG derived from WR45 or WR26 as described in section 11.4. This gave an HIU/HAU ratio of 3.75:1 and would ensure the neutralization of  $1 \times 10^7$  PFU FPV/R by >99.5%.

Virus- $[^{125}\text{I}]$ -labelled IgG complexes were sedimented through a 5 ml cushion of 5% sucrose in PBS by ultracentrifugation. The amount of radioactivity associated with the pellet was determined. Iodinated IgG binding to FPV/R was determined with respect to non-specific binding to influenza B/Lee. The number of IgG molecules binding was calculated from using a MW of IgG of 150,000; that 1 HAU FPV/R equals  $3.981 \times 10^7$  virus particles (v.p); there are an average of 1000 HA spikes per FPV/R particle and  $L = 6.02 \times 10^{23}$ /mol. Sp. act. of  $[^{125}\text{I}]$ -labelled WR45 =  $7.68 \times 10^4$  cpm/ug; sp act. of  $[^{125}\text{I}]$ -labelled WR26 =  $1.25 \times 10^5$  cpm/ug.

Sample calculation: Number of WR45 IgGs binding to each HA spike on FPV/R.

$$\frac{8452 \text{ cpm}}{7.6 \times 10^4 \text{ cpm}/\mu\text{g}} = 0.11 \mu\text{g IgG.}$$

$$\frac{0.11 \times 10^{-7} \text{ g}}{1.5 \times 10^5} = 7.33 \times 10^{-13}$$

$$7.33 \times 10^{-13} \times L = 4.42 \times 10^{11} \text{ molecules IgG.}$$

$$\frac{4.42 \times 10^{11}}{7.962 \times 10^8 \text{ v.p.}} = 555 \text{ IgG molecules per virus particle.}$$

$$= \underline{\underline{0.56 \text{ molecules per HA spike.}}}$$

Figure 13.1.1 and Figure 13.1.2 Number of specific polyclonal IgG molecules binding to each haemagglutinin spike on an intact FPV/R particle.

Figure 13.1.1 20 HAU FPV/R ( $7.962 \times 10^8$  particles) were incubated with increasing HIU of purified IgG (section 11.4) derived from WR45 (●—●) or WR26 (■—■). WR45 and WR26 were antisera produced from the inoculation of rabbits with FPV/D (H7N7) (section 10.7).

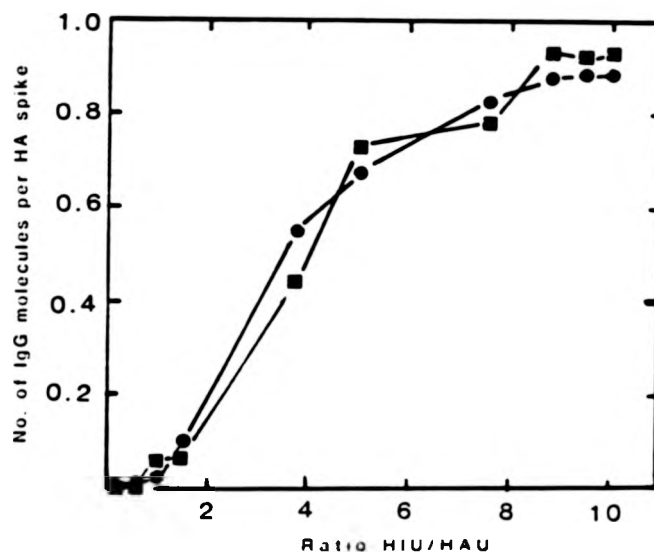
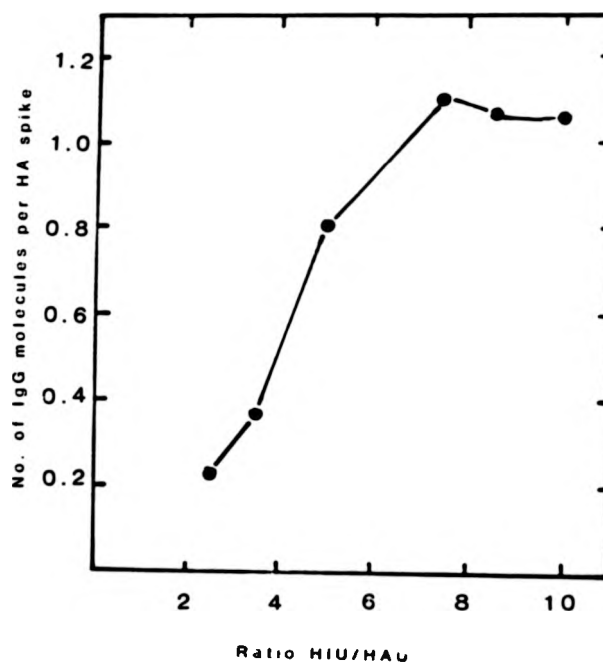


Figure 13.1.2.20 HAU FPV/R ( $7.962 \times 10^8$  particles) were incubated with increasing HIU of purified IgG (section 11.4) derived from WR69. WR69 was antiserum produced from the inoculation of rabbits (section 10.7) with V31 (H7N2) (section 10.15). The specific activity of [ $^{125}$ I]-labelled WR69 IgG was  $4.2 \times 10^4$  cpm/ug. The number of IgG molecules was calculated as described in Table 13.4.



(H7N1) (Figure 13.1.2).

The activity of IgG was expressed in terms of HIU because protein concentration and neutralization capability could not be reconciled with the variable degrees of iodination and inactivation inherent in producing [ $^{125}\text{I}$ ]-labelled antibody.

#### 13.4.2. Estimation of the number of radiolabelled monoclonal IgG molecules binding to FPV/R particles.

Binding curves for monoclonal antibodies HC2, HC10, HC58 and HC61 were also obtained (Figures 13.2.1 to 13.2.4). All showed that the maximum number of IgG molecules that would attach per spike was in the order of 1 (or 1000 IgG molecules per particle).

#### 13.4.3. The relationship between the number of antibodies binding to a FPV/R particle and neutralization.

Figure 13.3. shows the relationship between percentage neutralization of  $1.3 \times 10^4$  PFU and the number of HC2 IgG molecules bound per FPV/R particle. At 50% neutralization it was calculated that there were an average of 45 IgG molecules bound per particle (the "neutralization dose 50" or  $N_{50}$ ). To achieve more than 90% neutralization the virus population has to bind more than 150 IgG molecules/particle. Figure 13.4 demonstrates the number of HC10 IgG molecules that are required for



Figures 13.2.1-13.2.4. Number of monoclonal IgG molecules binding to HA spikes on FPV/R particles at increasing HIU/HAU ratios. Monoclonal IgG (derived from HC2, HC10, HC58 AND HC61) were incubated with 20 HAU FPV/R as described in section 11.4. and Table 13.4. The profiles of the binding of HC2 IgG (Fig. 13.2.1), HC10 IgG (Fig 13.2.2), HC58 IgG (Fig 13.2.3) and HC61 IgG (Fig. 13.2.4) indicate that the level of saturation is in the order of 1 IgG molecule per HA spike. The specific activities of [ $^{125}$ I]-labelled HC2, HC10, HC58 and HC61 were  $5.6 \times 10^4$ ,  $8.44 \times 10^4$ ,  $9.8 \times 10^4$  and  $5.14 \times 10^4$  cpm/ug respectively.

Figure 13.2.1. Number of HC2 IgG molecules binding

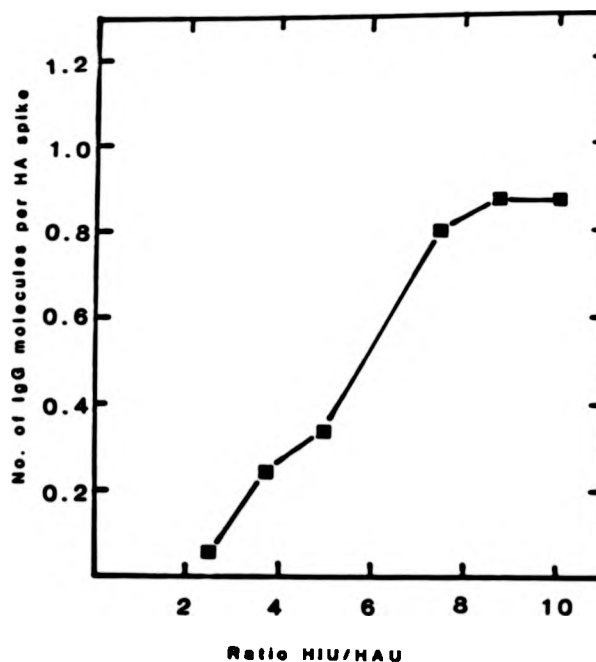


Figure 13.2.2. Number of HC10  
IgG molecules binding

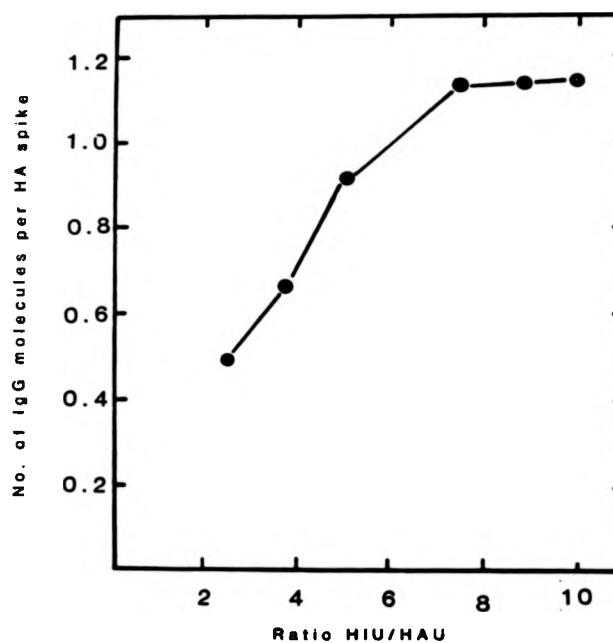


Figure 13.2.3. Number of HC58  
IgG molecules binding

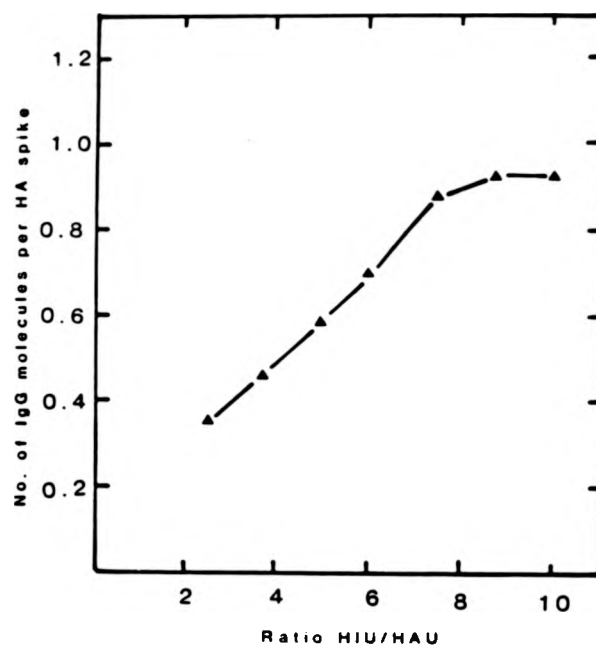
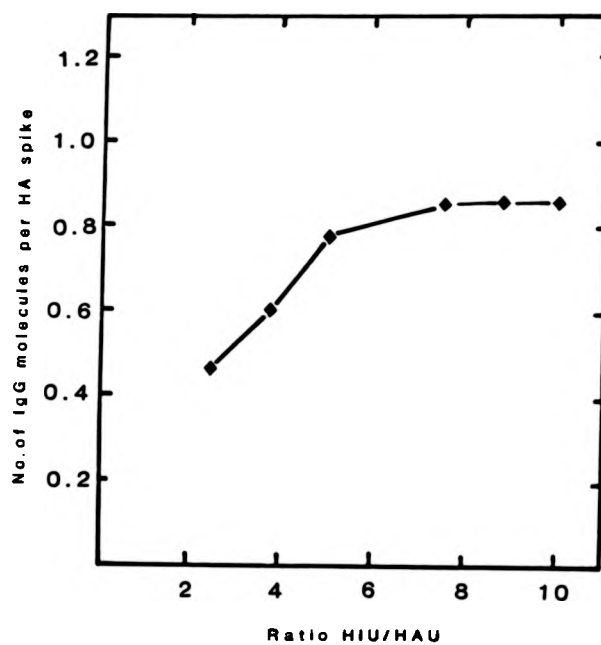


Figure 13.2.4. Number of HC61  
IgG molecules binding



neutralization of  $2 \times 10^7$  PFU FPV/R. The  $N_{50}$  for HC10 was 50 IgG molecules per particle which compares favourably with the  $N_{50}$  of HC2 of 45. The relationship between the number of IgG molecules binding and neutralization is linear up to 99.98% neutralization.

Figure 13.3. Graph showing the percentage neutralization against the number of monoclonal (HC2) IgG molecules binding to a FPV/R particle. The  $N_{50}$  is the amount of IgG binding that will neutralize 50% of the virus, i.e. 45 IgG molecules/particle. The number of IgG molecules was calculated as described in Table 13.4.

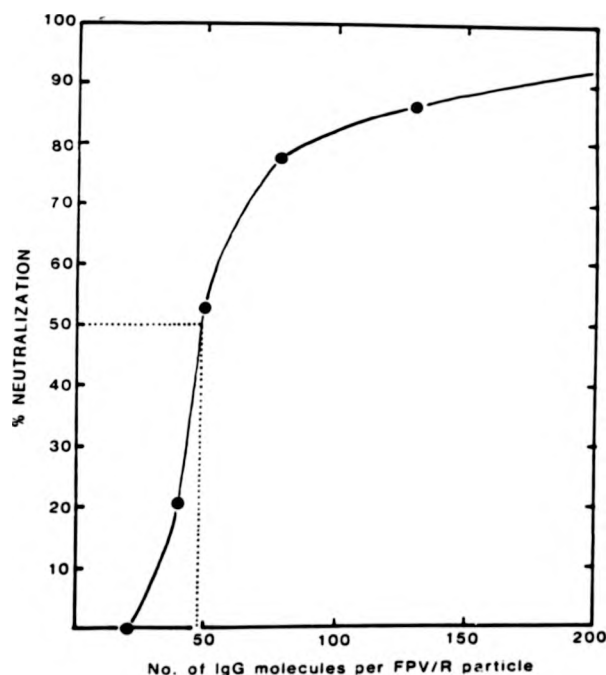


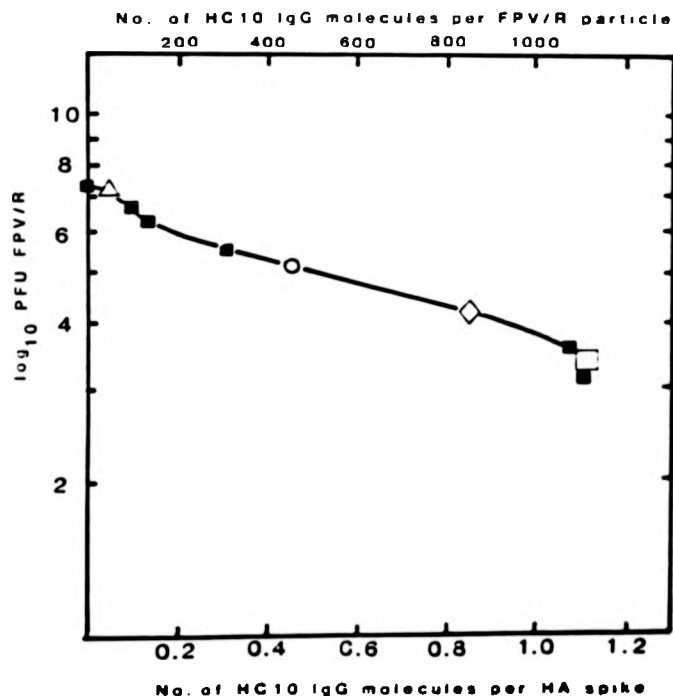
Figure 13.4. Reduction in the infectivity of  $2 \times 10^7$  PFU FPV/R by HC10 IgG correlated with the number of IgG molecules binding per HA spike or FPV/R particle. Increasing amounts of [ $^{125}$ I]-labelled HC10 were incubated with  $2 \times 10^7$  PFU and the % neutralization measured by plaque reduction (section 10.11). Parallel binding assays (section 11.4) were carried out in order to estimate the number of IgG molecules bound/particle (■). Significant neutralization titres are denoted:

50% :  $\triangle$

99% :  $\circ$

99.9% :  $\diamond$

99.98% :  $\square$



### 13.5. Two step competition assay of binding IgG to influenza virus.

These experiments were carried out using two different populations of purified immunoglobulins incubated separately with 20 HAU FPV/R. These investigations would determine if the feature of not more than one antibody binding to a HA spike was a functional or an intrinsic phenomenon. The monoclonal IgGs used in each assay mapped to different epitopes, therefore direct competition should not be a factor in the inhibition of binding. Prior to each experiment in these studies binding curves for each [ $^{125}$ I]-labelled IgG were obtained and saturating levels (in terms of HIU) were determined. Using this information two step incubation assays of 20 HAU FPV/R with unlabelled and labelled immunoglobulins were performed. The maximum number of IgG molecules binding per spike was consistently in the order of 1 (mean value for all IgGs at 175 HIU = 1.1,  $\pm$  0.28). All antibodies exhibited homologous inhibition, i.e. unlabelled IgG inhibited the binding of labelled IgG from the same source.

#### 13.5.1. HC58 (mapping to epitope Z) and HC10 (mapping to epitope Y) IgG.

Firstly, FPV/R was incubated with increasing amounts of unlabelled HC58 at 2.5, 3.75, 5 and 8.75 HIU/HAU ratios for 1h at 25°C, and then for 18h at 4°C. After this time a saturating



**Figure 13.5.** Increasing amounts of unlabelled HC58 (mapping to epitope Z) IgG (from 50-180 HIU) restrict the binding of a constant amount (175 HIU) of [ $^{125}$ I]-labelled HC10 (mapping to epitope Y) IgG. 20 HAU FPV/R was incubated with increasing HIU of unlabelled HC58 before a second incubation with 175 HIU [ $^{125}$ I]-labelled HC10 IgG (section 13.5.1). Specific activity of [ $^{125}$ I]-labelled HC10 IgG =  $9.07 \times 10^4$  cpm/ug, specific activity of [ $^{125}$ I]-labelled HC58 IgG =  $9.8 \times 10^4$ /ug. The binding of [ $^{125}$ I]-labelled HC10 IgG (●●●●) was reduced from 1.1 IgG molecules per spike (iodinated HC10 IgG alone with virus.●.....) to 0.03 per spike.

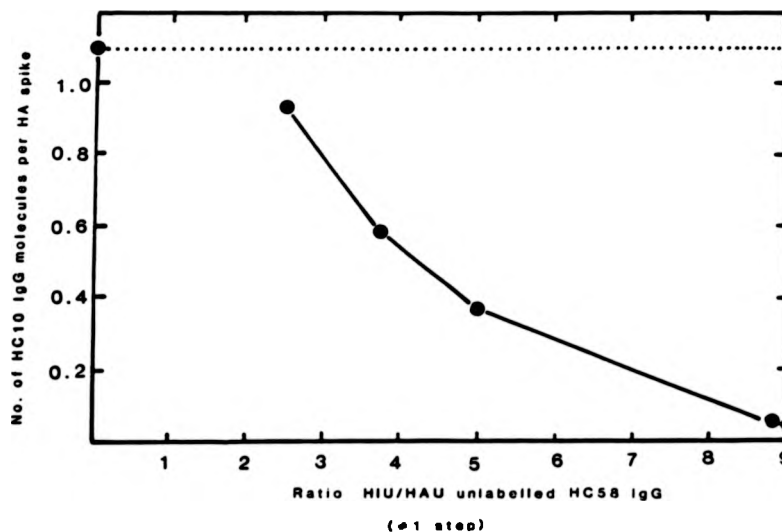
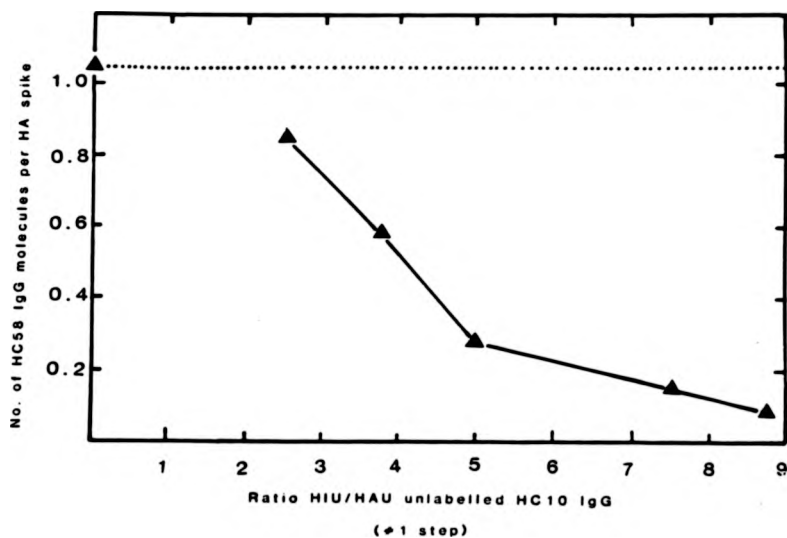


Figure 13.6. Increasing amounts of unlabelled HC10 IgG (from 50-175 HIU) restrict the binding of a constant amount (175 HIU) of [ $^{125}$ I]-labelled HC58 IgG. Experiment performed as described in Figure 13.5, except that incubation of unlabelled HC10 IgG was followed by incubation with [ $^{125}$ I]-labelled HC58 IgG. The binding of [ $^{125}$ I]-labelled HC58 IgG ( $\blacktriangle$ ) was reduced from 1.05 IgG molecules per spike (iodinated HC58 IgG alone with virus,  $\blacktriangle$ .....) to 0.09 per spike.



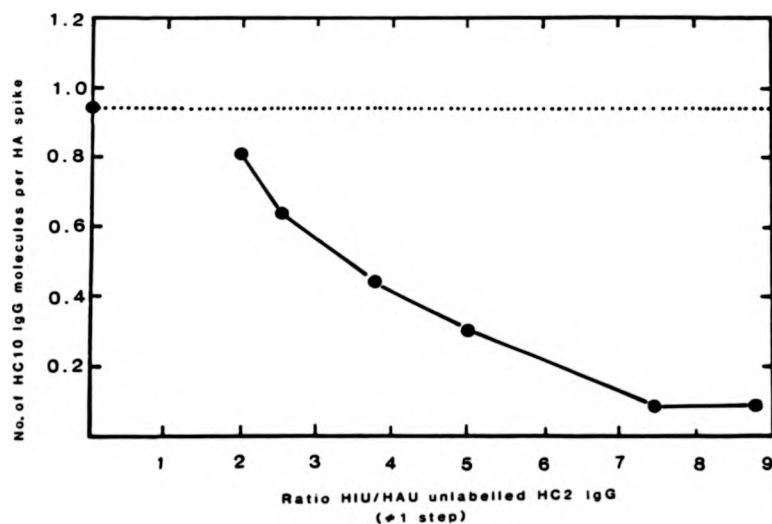
amount (8.75 HIU/HAU) of [ $^{125}$ I]-labelled HC10 IgG was added to each virus:antibody mixture. The radioactivity associated with the virus pellet was determined (section 11.4) and Figure 13.5. shows that increasing the concentration of unlabelled HC58 restricted the attachment of [ $^{125}$ I]-labelled HC10 IgG from 1.1 antibody per HA spike to less than 0.03 per spike.

Figure 13.6 illustrates that this pattern was essentially repeated when the incubations were reversed using increasing amounts of unlabelled HC10 IgG against a second incubation of a constant amount of [ $^{125}$ I]-labelled HC58 IgG that would without the HC10 incubation achieve saturation. The binding of [ $^{125}$ I]-labelled HC58 IgG was reduced from 1.05 IgG molecules per spike to 0.09 per spike. Thus, despite these antibodies being directed to different epitopes they prevented the binding of the second antibody, indicating that inhibition could be steric hindrance and is intrinsic to the binding of antibody to HA spikes.

#### 13.5.2. HC2 (mapping to epitope Z) and HC10 (mapping to epitope Y) IgG.

This experiment was performed as described in section 13.5.1. except that HC2 was used instead of HC58. Both HC58 and HC2 map to antigenic site Z (section 13.3.1; Table 13.2). Figure 13.7 shows that increasing the concentration of unlabelled HC2 restricted the attachment of [ $^{125}$ I]-labelled HC10 IgG from 0.94 antibody per HA spike to less than 0.09 per spike. A reciprocal

Figure 13.7. Increasing amounts of unlabelled HC2 (mapping to epitope Z) IgG (from 50-175 HIU) restrict the binding of a constant amount (175 HIU) of [ $^{125}$ I]-labelled HC10 (mapping to epitope Y) IgG. Experiment performed as described in Figure 13.5, except that incubation of unlabelled HC2 IgG was followed by incubation with [ $^{125}$ I]-labelled HC10 IgG. The binding of [ $^{125}$ I]-labelled HC10 IgG (●—●) was reduced from 0.94 IgG molecules per spike (iodinated HC58 IgG alone with virus, ●.....) to 0.09 per spike. The specific activity of [ $^{125}$ I]-labelled HC10 IgG =  $8.44 \times 10^4$  cpm/ug.



experiment with unlabelled HC10 and labelled HC2 produced a similar result.

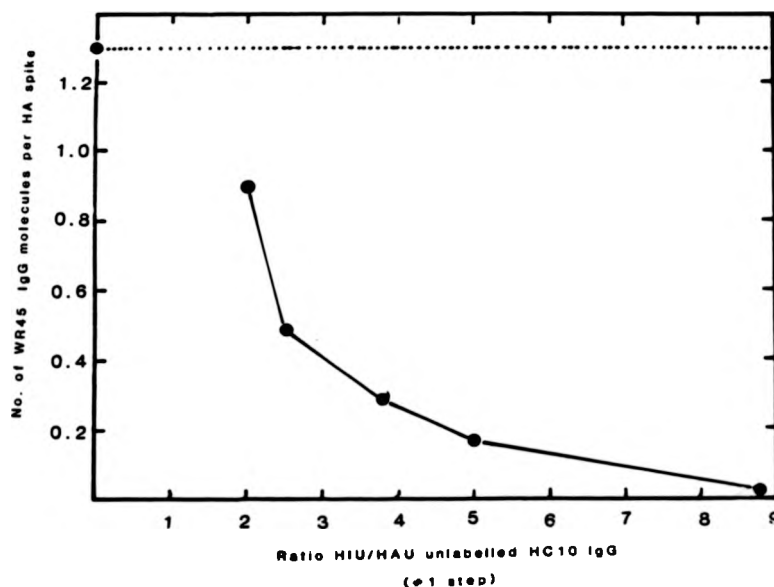
These studies indicate that the initial incubation of increasing concentrations of unlabelled monoclonal antibody restricts, almost completely the binding of the other antibodies tested. This consistently occurs despite the fact that the two monoclonal IgGs used in each assay map to different epitopes.

In the next series of experiments the ability of monoclonal antibodies to prevent binding of polyclonal IgG (WR45 or WR69) was investigated.

#### 13.5.3. HC10 (mapping to epitope Y) and WR45 (polyclonal) IgG.

WR45 is a polyclonal IgG and would be expected to contain a mixed population of IgG molecules binding to a range of neutralizing epitopes. In the two step incubation assay it was found that preliminary incubation of unlabelled HC10 in increasing amounts (2-8.75 HIU/HAU) with 20HAU FPV/R restricted the attachment of a constant amount (8.75 HIU/HAU) of [ $^{125}$ I]-labelled WR45 IgG in the second incubation (Figure 13.8). This amount of WR45 would be expected to bind in the order of 1 IgG per spike, but in fact was restricted from 1.3 to an undetectable number of molecules per spike by increasing amounts of HC10.

Figure 13.8. Increasing amounts of unlabelled HC10 (mapping to epitope Y) IgG (from 40-175 HIU) restrict the binding of a constant amount (175 HIU) of [ $^{125}$ I]-labelled WR45 IgG. Experiment performed as described in Figure 13.5, except that incubation of unlabelled HC10 IgG was followed by incubation with [ $^{125}$ I]-labelled polyclonal WR45 IgG. The binding of [ $^{125}$ I]-labelled WR45 IgG (●—●) was reduced from 1.3 IgG molecules per spike (iodinated WR45 IgG alone with virus, ●.....) to <0.01 per spike. The specific activity of [ $^{125}$ I]-labelled WR45 IgG =  $2.84 \times 10^4$  cpm/ug.



#### 13.5.4. HC2 (mapping to epitope Z) and WR45 (polyclonal) IgG.

Figure 13.9 shows that if unlabelled HC2 IgG is incubated first with 20 HAU FPV/R at increasing levels (from 40-175 HIU) then it restricts the binding of [ $^{125}$ I]-labelled WR45 IgG molecules down to 0.06 per HA spike. At the concentration of WR45 used and without the preliminary incubation with HC2 IgG it would be expected that WR45 IgG would bind at a level of 1.15 IgG molecules per HA spike.

The inhibition of binding by HC2 is especially significant when the properties of WR45 are considered. Antigenic variant studies demonstrated (section 13.3.2) that WR45 on incubation with FPV/R can select mutants that are resistant to HI by HC10. Thus antigenic site Y would seem to be a crucial site as far as WR45 is concerned and direct competition could have been the reason for the inhibition of binding. However, HI and neutralization studies have shown that WR45 also contains other populations of IgG (Tables 13.2; 13.3) that interact with FPV/R HA at epitopes X and Z whose binding is inhibited by HC2.

#### 13.5.5. HC2 (mapping to epitope Z) and WR69 (polyclonal) IgG.

WR69 IgG is polyclonal and its anti H7 response is directed against the H7 of V31 (section 10.15) which was donated by FPV/R. Figure 13.10 shows that if unlabelled HC2 IgG is incubated first

Figure 13.9. Increasing amounts of unlabelled HC2 (mapping to epitope Z) IgG (from 40-175 HIU) restrict the binding of a constant amount (175 HIU) of [ $^{125}$ I]-labelled WR45 IgG. Experiment performed as described in Figure 13.5, except that incubation of unlabelled HC2 IgG was followed by incubation with [ $^{125}$ I]-labelled WR45 polyclonal IgG. The binding of [ $^{125}$ I]-labelled WR45 IgG (●—●) was reduced from 1.15 IgG molecules per spike (iodinated WR45 IgG alone with virus, ●.....) to 0.06 per spike. The specific activity of [ $^{125}$ I]-labelled WR45 IgG =  $2.94 \times 10^4$  cpm/ug.

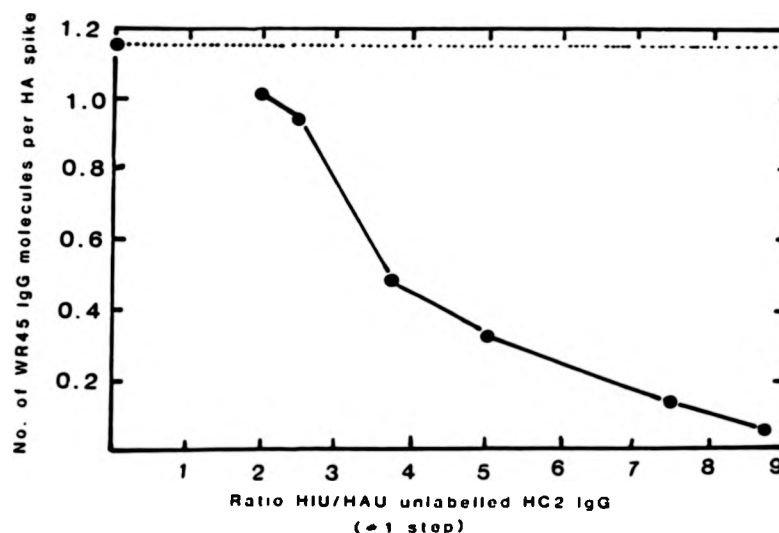
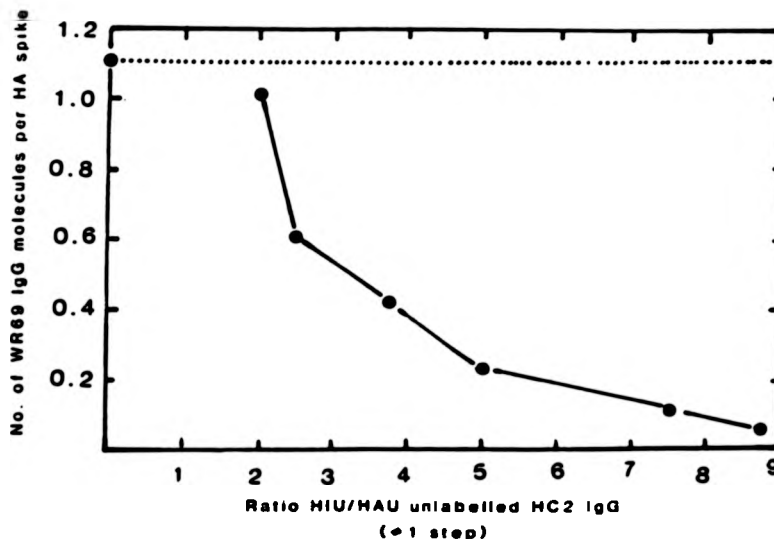




Figure 13.10. Increasing amounts of unlabelled HC2 (mapping to epitope Z) IgG (from 40-175 HIU) restrict the binding of a constant amount (175 HIU) of [ $^{125}$ I]-labelled WR69 IgG. Experiment performed as described in Figure 13.5, except that incubation of unlabelled HC2 IgG was followed by incubation with [ $^{125}$ I]-labelled WR69 polyclonal IgG. The binding of [ $^{125}$ I]-labelled WR69 IgG (●—●) was reduced from 1.11 IgG molecules per spike (iodinated WR69 IgG alone with virus, ●.....) to 0.04 per spike. The specific activity of [ $^{125}$ I]-labelled WR69 IgG =  $4.22 \times 10^4$  cpm/ug.



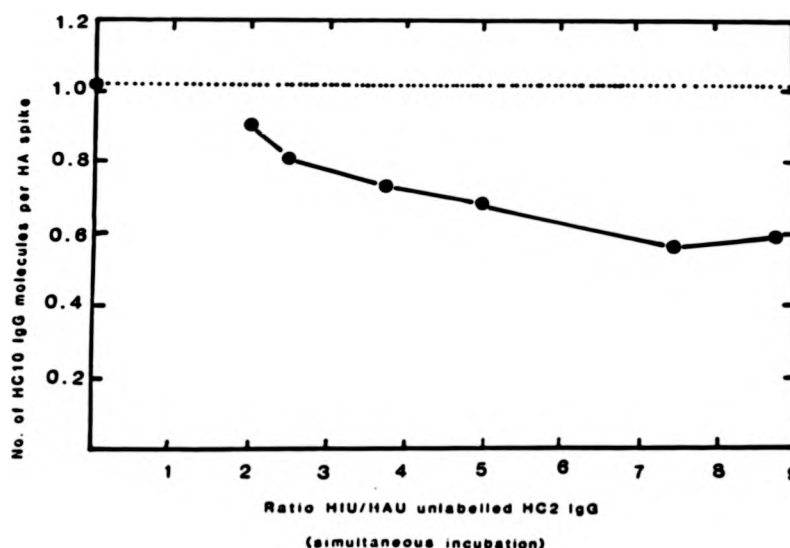
with 20 HAU FPV/R at increasing levels (from 40-175 HIU) then it restricts the binding of [ $^{125}$ I]-labelled WR69 IgG molecules down to 0.04 IgG molecules per HA spike. At the concentration of WR69 used and without the preliminary incubation with HC2 IgG it would be expected that WR69 IgG would bind at a level of 1.11 IgG molecules per HA spike. This experiment shows that the restriction of binding by monoclonal antibody is not limited to one source of polyclonal IgG.

13.6. Competition assay with HC2 (mapping to epitope Z)  
and HC10 (mapping to epitope Y) IgG.

This series of experiments, performed with HC2 and HC10 IgG, was designed to find out how the simultaneous incubation of two monoclonal IgG populations affected the amount of binding. In earlier experiments both HC2 and HC10 bound to a maximum of approximately one IgG per HA spike. Also in two step incubation experiments each reciprocally inhibited the binding of the other.

Different results were achieved in a direct competition assay with simultaneous incubation of antibody and virus. In Figure 13.11 increasing the concentration of unlabelled HC2 restricted the attachment of [ $^{125}$ I]-labelled HC10 IgG to only 0.56-0.59 IgG molecule per spike in contrast to the results in a two step incubation assay where binding was restricted to less than 0.1 (Figure 13.7).

Figure 13.11. Competition assay to show how the presence of increasing amounts of HC2 (mapping to epitope Z) IgG (50-200 HIU) affects the binding of [ $^{125}$ I]-labelled HC10 (mapping to epitope Y) IgG in simultaneous incubation. Increasing amounts of unlabelled HC2 IgG (from 40-175 HIU) were added simultaneously with a constant amount of [ $^{125}$ I]-labelled HC10 (175 HIU) to 20 HAU FPV/R (section 13.6). In this manner the binding of HC10 IgG was only restricted to 0.56-0.59 IgG molecules per spike. The specific activity of [ $^{125}$ I]-labelled HC10 IgG =  $8.44 \times 10^4$  cpm/ug.



These levels of approximately 0.5 IgG molecules per spike would be expected in an assay that allowed two IgG antibodies with similar affinities to bind with equal opportunity. It could be pictured from these data that at the end of the incubation period the haemagglutinin spikes would have an average of 1 IgG molecule per spike with one in two spikes having a [ $^{125}\text{I}$ ]-labelled HC10 IgG bound.

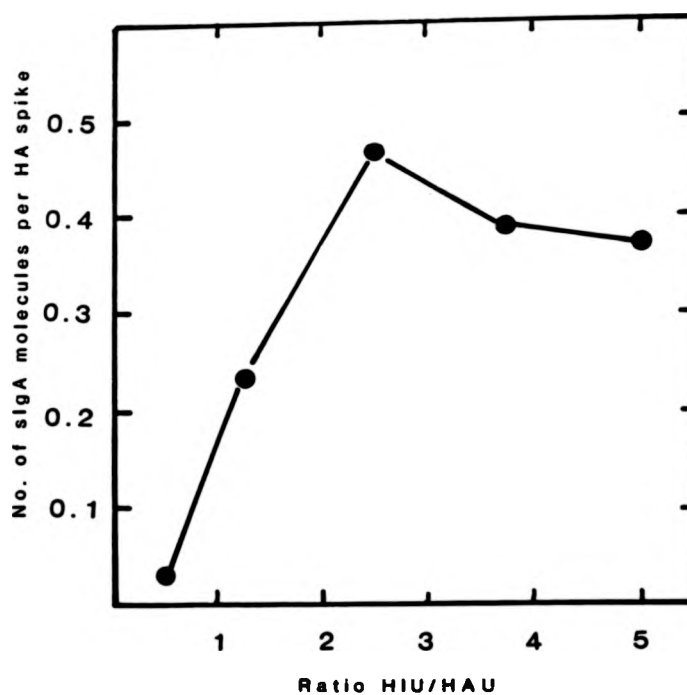
#### 13.7. Number of polyclonal sIgA molecules able to bind to an HA spike of FPV/R.

The binding curve obtained for polyclonal sIgA performed as described in section 11.4 showed that the maximum number able to bind was in the order of 0.4 molecules per HA spike (Figure 13.12). An sIgA molecule consists of two four chain immunoglobulin units (section 6.2.3), therefore 0.4 sIgA is approximately equivalent to 0.8 immunoglobulin units. At the concentration of 100 HIU sIgA to 20 HAU FPV/R the level of neutralization of  $1 \times 10^7$  PFU was 97.5%.

##### 13.7.1. Two step binding assay with polyclonal sIgA and HC81 IgG (mapping to epitope X).

In a two step assay a constant saturating amount of unlabelled sIgA (3.75 HIU/HAU) was first incubated with 20 HAU

Figure 13.12. Number of polyclonal sIgA molecules binding to HA spikes on FPV/R particles at increasing HIU/HAU. Polyclonal sIgA (purified by gel filtration) was incubated with 20 HAU (as described in section 11.4 and Table 13.4). The profiles of the binding of sIgA indicates that the level of saturation is in the order of 0.4 HA per spike. The number of sIgA molecules binding was calculated from using a MW of sIgA of 400,000. The specific activity of [ $^{125}$ I]-labelled sIgA was  $1.463 \times 10^4$  cpm/ $\mu$ g.



**Figure 13.13.** Unlabelled polyclonal sIgA (75 HIU) restricts the binding of increasing amounts of [ $^{125}$ I]-labelled HC61 IgG. 20 HAU FPV/R was incubated with a constant amount (giving an HIU/HAU ratio of 3.75) of sIgA before a second incubation with 100-175 HIU of [ $^{125}$ I]-labelled HC61 IgG (mapping to antigenic site X). The binding of HC61 was restricted to below 0.18 IgG molecules per HA spike (◆—◆) when incubation of HC61 IgG alone with virus at such levels achieved >0.8 IgG molecules per HA spike (◇---◇). The specific activity of [ $^{125}$ I]-labelled HC61 IgG =  $5.14 \times 10^4$  cpm/ug.

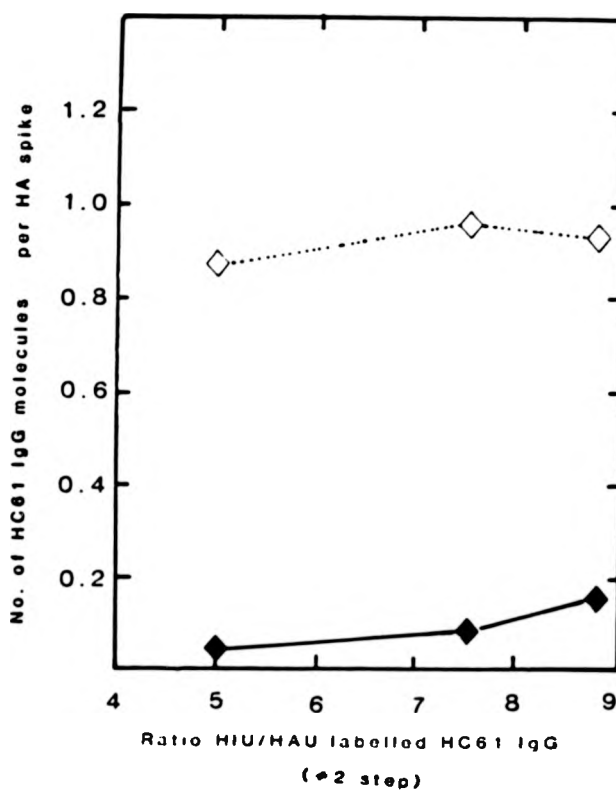
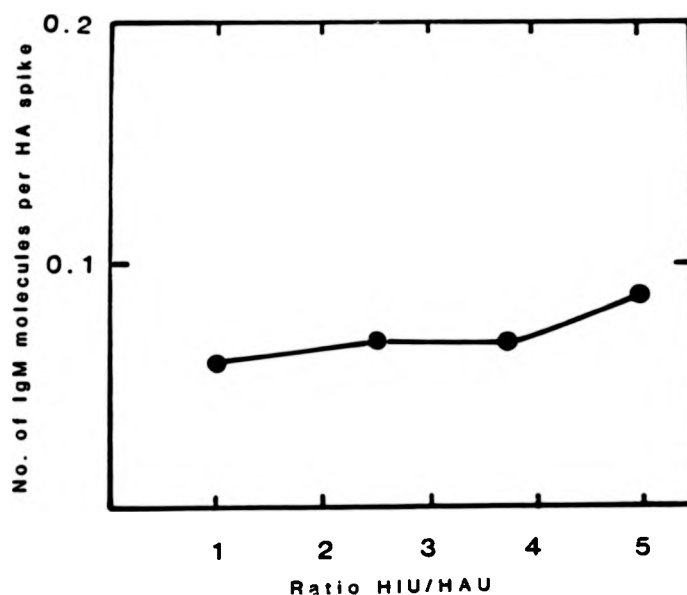


Figure 13.14. Number of polyclonal IgM molecules binding to HA spikes on FPV/R particles at increasing HIU/HAU. Polyclonal IgM (purified by gel filtration) was incubated with 20 HAU (as described in section 11.4 and Table 13.4). The profiles of the binding of IgM indicates that the level of saturation is in the order of  $<0.1$  HA per spike. The number of IgM molecules binding was calculated from using a MW of IgM of 900,000. The specific activity of  $[^{125}\text{I}]$ -labelled IgM was  $9.7 \times 10^3$  cpm/ug. 100 HIU per 20 HAU achieved 98.5% neutralization.



before incubating with increasing amounts of [ $^{125}\text{I}$ ]-labelled HC61 IgG was added (at 5, 7.5 and 8.75 HIU/HAU). Figure 13.13 shows that the binding of HC61 was restricted to below 0.2 IgG molecules per spike by the previous incubation with sIgA.

#### 13.8. Number of polyclonal IgM molecules able to bind to the HA spikes of FPV/R.

The binding curve obtained for polyclonal IgM showed that the maximum number able to bind was in the order of 0.1 per haemagglutinin spike (Figure 13.14). At the concentration of 100 HIU IgM to 20 HAU FPV/R the level of neutralization of  $1 \times 10^7$  PFU was 98.5%.

#### 13.9. Binding of IgG in solid-phase radioimmunoassay.

These assays differed from the previous assays in that the antigen, FPV/R particles (5 HAU) and BrHA (HA enzymatically removed from FPV/R particles), were adsorbed to the wells of plastic microtitre plates.

Binding curves obtained with HC2 and HC10 in solid-phase RIA (Figures 13.15.1; 13.15.2) showed that in the order of 3 IgG molecules bind per HA spike. Essentially the same curves for HC2 and HC10 was seen whether FPV/R particles or BrHA molecules were used as the solid-phase binding substrate.



Figure 13.15.1. and Figure 13.15.2. Number of HC2 IgG and HC10 IgG molecules binding to FPV/R particles (5 HAU/well) (●) or to the equivalent HA of isolated BrHA spikes (■) in solid phase RIA. 5 HAU intact FPV/R or isolated BrHA were adsorbed onto the surfaces of microtitre plate wells (section 11.4.2).

Figure 13.15.1. Increasing amounts of [ $^{125}$ I]-labelled HC2 IgG (5-90 HIU) were added to prepared microtitre wells for 1 h at 25°C and then 18 h at 4°C. The wells were then washed thoroughly with cold PBS and the radioactivity associated with the wells determined allowing the number of IgG molecules binding to be calculated. The specific activity of [ $^{125}$ I]-labelled HC2 IgG =  $4.95 \times 10^4$  cpm/ug.

Figure 13.15.2. Increasing amounts of [ $^{125}$ I]-labelled HC10 IgG (5-90 HIU) was added to prepared microtitre wells as described in Figure 13.15.1. The specific activity of [ $^{125}$ I]-labelled HC10 IgG =  $4.28 \times 10^4$  cpm/ug.

Figure 13.15.1.

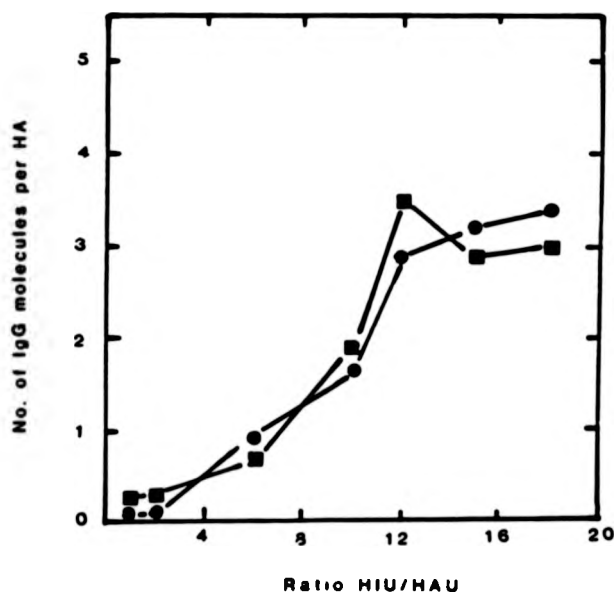


Figure 13.15.2.

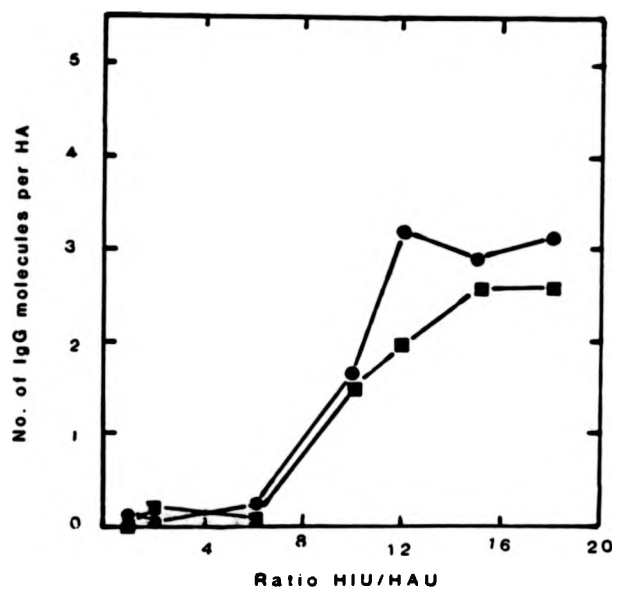


Figure 13.16. Increasing amounts of unlabelled HC10 IgG (10-75 HIU) restrict the binding of constant amount (75 HIU) of [ $^{125}$ I]-labelled HC2 IgG (■—■). Experiment performed as described in Figure 13.15.1 except that incubation of increasing amounts (10-75 HIU) of unlabelled HC10 IgG was followed by incubation with 75 HIU [ $^{125}$ I]-labelled HC2 IgG in microtitre wells coated with intact FPV/R particles at 5 HAU per well. The binding of a constant amount of [ $^{125}$ I]-labelled HC2 IgG was reduced from 3.85 IgG molecules per HA spike (iodinated HC2 IgG alone with virus, ■... ) to 0.05 per spike.

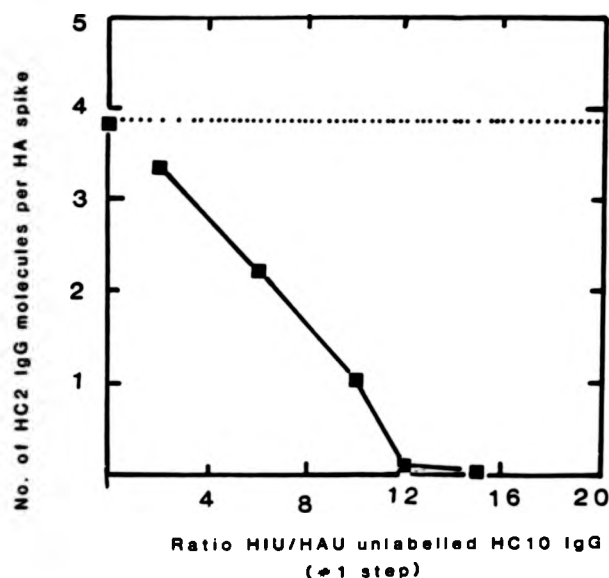
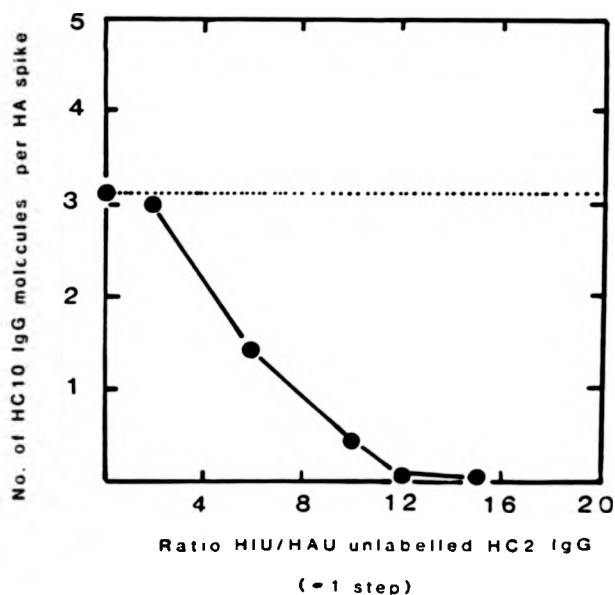


Figure 13.17. Increasing amounts of unlabelled HC2 IgG (10-75 HIU) restrict the binding of a constant amount (75 HIU) of [ $^{125}$ I]-labelled HC10 IgG (●—●). Experiment performed as described in Figure 13.15.1 except that incubation of increasing amounts (10-75 HIU) of unlabelled HC2 IgG was followed by incubation with 75 HIU [ $^{125}$ I]-labelled HC10 IgG in microtitre wells coated with intact FPV/R particles at 5 HAU per well. The binding of a constant amount of [ $^{125}$ I]-labelled HC10 IgG was reduced from 3.1 IgG molecules per HA spike (iodinated HC10 IgG alone with virus, ●.....) to <0.05 per spike.



In two step incubation assays it was found (Figures 13.16; 13.17) that increasing amounts of unlabelled antibody (either HC2 or HC10 IgG) in the first incubation with 5 HAU FPV/R restricted the binding of a constant amount of [ $^{125}$ I]-labelled IgG (either HC10 or HC2) from 3 IgG molecules per spike to an almost undetectable level.

Thus the use of a solid-phase RIA results in an increase in the number of IgG molecules able to bind from 1 per HA spike observed with FPV/R particles free in solution to approximately three. In the solid-phase RIA restriction of binding of a second antibody occurs in much the same way as that when virus is free in suspension, i.e. three IgGs binding per HA spike are able to inhibit the binding of further antibody to theoretically available antigenic regions.

#### 13.10. Kinetic studies on the interaction between FPV/R and monoclonal IgG.

Figure 13.18 is a plot of the course of inactivation of influenza virus by purified monoclonal HC2 IgG as a function of time. At this temperature (25°C) and concentration (0.01  $\mu$ g IgG), there is an immediate loss of virus infectivity which could be taken as evidence for the inactivation of one influenza virus particle by one antibody molecule (section 8.1.1; Figure 8.1). Figure 13.19 shows that the slope of the curve is dependent on

Figure 13.18. Kinetics of inactivation of 60 PFU FPV/R by purified monoclonal HC2 IgG (0.01 $\mu$ g). Virus and IgG were mixed at  $t=0$  and incubated at 25°C. At subsequent times samples were inoculated onto CEF monolayers in duplicate and assayed for residual infectivity (section 10.1).

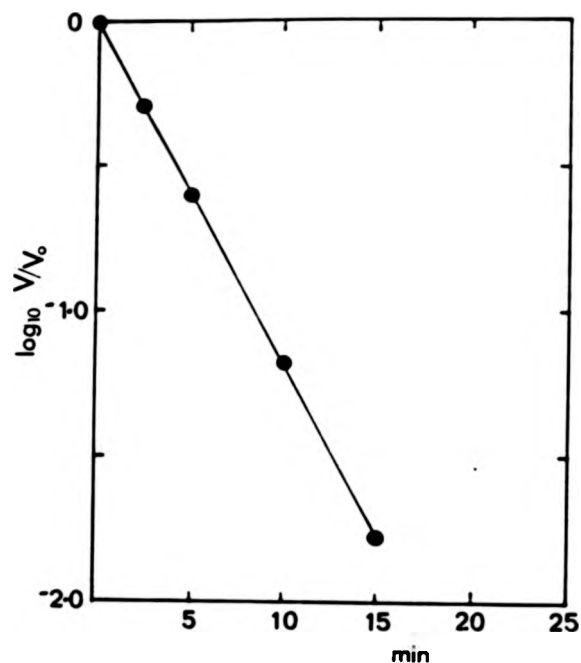
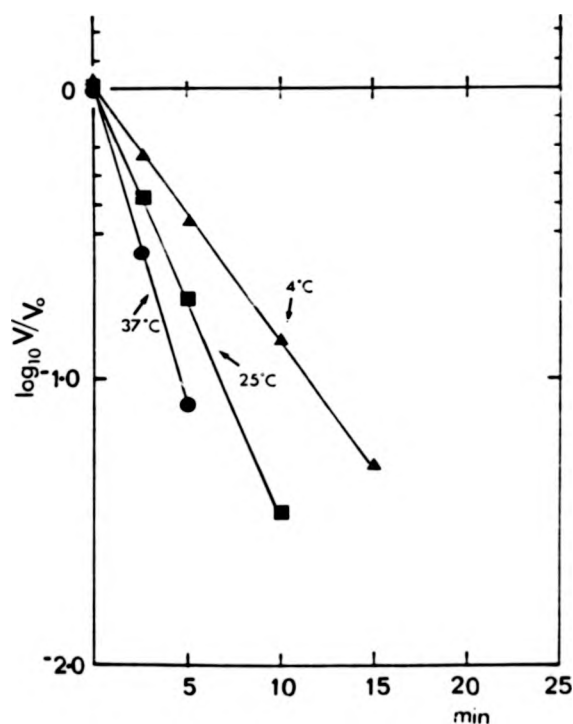


Figure 13.19. Kinetics of inactivation of 60 PFU FPV/R by purified monoclonal HC2 IgG (0.1 $\mu$ g) at 4, 25, or 37°C. Virus and IgG were mixed at t=0 and incubated at either 4°C (▲), 25°C (■), or 37°C (●). At subsequent times samples were inoculated onto CEF monolayers in duplicate and assayed for residual infectivity (section 10.1). The intercept of the lines of best fit calculated by linear regression analysis are shown (correlation coefficients for 37, 25, and 4°C were 0.99, 0.99, and 0.97 respectively).



the temperature; decreasing the temperature whilst keeping the antibody concentration constant (0.1  $\mu\text{g}$ ) decreased the rate of virus inactivation. Decreasing the IgG concentration to 0.01  $\mu\text{g}$  IgG per 60 PFU at 4°C produced a slight lag in the loss of FPV/R survival with both HC2 and HC58 IgG (Figures 13.20; 13.21 respectively). Linear regression analysis and extrapolation of the slope obtained with 0.01 $\mu\text{g}$  HC2 indicated that 3 HC2 IgG molecules were required for neutralization. Similar analysis of the slope achieved with 0.01  $\mu\text{g}$  HC58 IgG indicated that 3.4 (or between 3 and 4) IgG molecules were required. An indication of a lag phase was seen with 0.03  $\mu\text{g}$  HC58 IgG. linear regression analysis and extrapolation of the slope predicted that 2.8 (i.e 3) IgG molecules were required. At these concentrations shoulders were not produced when virus and antibody were incubated at 25 and 37°C. Reducing the concentration of both antibodies below 0.01  $\mu\text{g}$  in this system produced variable results.

The lag phase observed at 4°C with low concentrations of IgG is compatible with the hypothesis that the neutralization of FPV/R required the binding of more than one IgG molecule with a virus particle to bring about neutralization (Lafferty, 1963a) and is thus a multi-hit phenomenon.



Figure 13.20. Kinetics of inactivation of 60 PFU FPV/R by 0.1, 0.03, and 0.01  $\mu\text{g}$  purified monoclonal HC2 IgG at 4°C. Virus and either 0.1  $\mu\text{g}$  (●), 0.03  $\mu\text{g}$  (▲), or 0.01  $\mu\text{g}$  (■) IgG were mixed at  $t=0$  and incubated at 4°C. At subsequent times samples were inoculated onto CEF monolayers in duplicate and assayed for residual infectivity (section 10.1). The intercept of the lines of best fit calculated by linear regression analysis are shown (correlation coefficients for 0.1, 0.03, and 0.01  $\mu\text{g}$  were 0.99, 0.99, and 0.96 respectively).

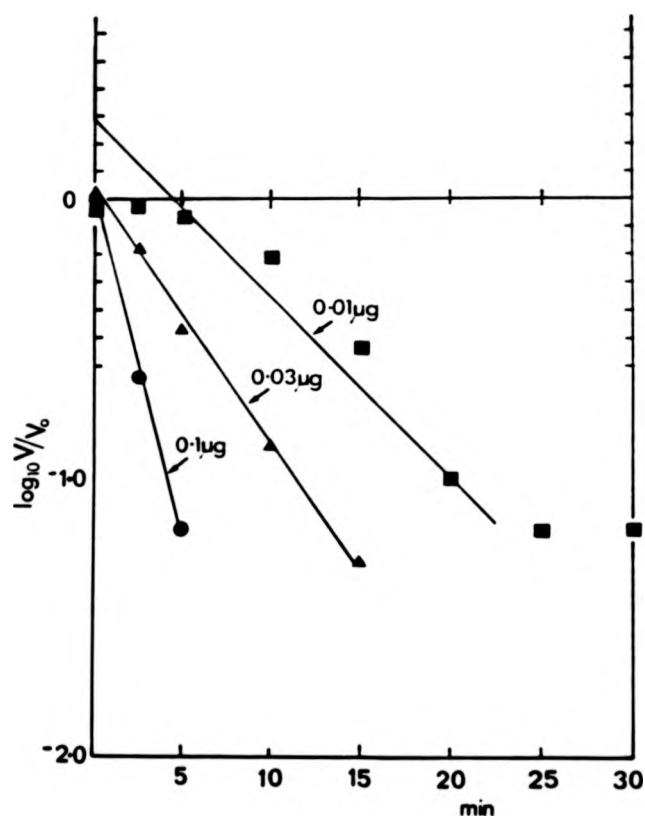
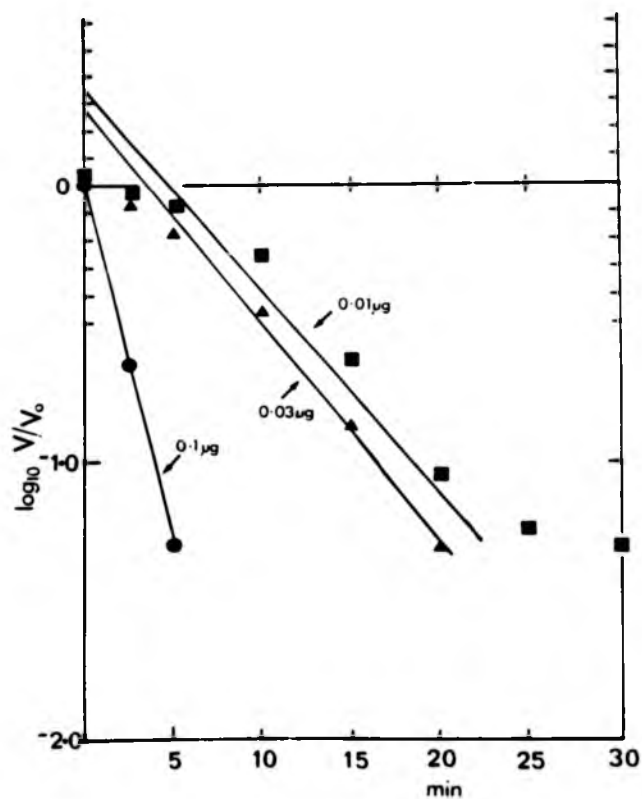


Figure 13.21. Kinetics of inactivation of 60 PFU FPV/R by 0.1, 0.03, and 0.01  $\mu\text{g}$  purified monoclonal HC58 IgG at 4°C. Virus and either 0.1  $\mu\text{g}$  (●), 0.03  $\mu\text{g}$  (▲), or 0.01  $\mu\text{g}$  (■) IgG were mixed at  $t=0$  and incubated at 4°C. At subsequent times samples were inoculated onto CEF monolayers in duplicate and assayed for residual infectivity (section 10.1). The intercept of the lines of best fit calculated by linear regression analysis are shown (correlation coefficients for 0.1, 0.03, and 0.01  $\mu\text{g}$  were 0.99, 0.93, and 0.95 respectively).



### 13.11. DISCUSSION

#### 13.11.1. The average number of HA spikes on a FPV/R particle is 1000.

The investigations in this section rely on an accurate determination of the average number of HA spikes on an FPV/R particle. Electron microscopy was employed to estimate the number of surface projections, following the method employed by Tiffany and Blough (1970b) for myxo- and paramyxoviruses. The number of surface projections is calculated from the mean surface area of virus particles (calculated from the diameter) and the mean area occupied by each spike (calculation given in section 13.4.1). The average diameter of quasispherical FPV/R virus particles was determined to be 120 nm, which compares favourably with the average diameter of 118 nm determined for the reassortant influenza A virus V31 (section 10.15) by small angle X-ray scattering (M.T. Stubbs and H.P. Taylor, unpublished data) and the range of diameters estimated in EM studies for influenza A virus of 80-150 nm (Wrigley, 1979). By neutron scattering the average diameter of laboratory passaged strains of influenza A viruses was determined to be 120 nm (Cusack, 1982). The mean distance separating spikes of 5.1 nm used in my calculation is at the narrow end of the range of 5-8 nm reported for influenza A viruses (Tiffany and Blough, 1970b). The calculated mean number of spikes per FPV/R particle was 1273 (section 13.1), which is at the top of the reported range for a number of (mostly) human influenza A viruses. The proportion of HA to NA was determined (section 13.2) to be 4.6/1, which is within the reported range of

4/1 to 5/1 (Wrigley, 1979). Therefore the average number of HA spikes on a FPV/R particle was taken to be approximately 1000.

**13.11.2. FPV/R in suspension is saturated with approximately one IgG per HA spike.**

Knowing the specific activity of radiolabelled immunoglobulins and knowing the total radioactivity bound per particle means that it is possible to estimate the number of immunoglobulin molecules bound to each HA spike on intact FPV/R particles. Errors inherent to such measurements mean that the estimates of the number of immunoglobulin molecules binding should not be taken as precise values, rather as a indication of the order of magnitude.

Saturation of FPV/R with HC2, HC10, HC58 and HC61 [ $^{125}\text{I}$ ]-labelled IgG showed consistently that only one IgG molecule could bind to each FPV/R HA spike. Polyclonal IgG (WR26 and WR45) also bound up to a maximum of 1 IgG molecule per HA spike. It appears that the binding of one IgG molecule to a HA spike precludes any further immunoglobulin molecules from binding. Therefore we have the situation that a monoclonal antibody bound to a HA spike prevents the binding of any more monoclonal antibody to the other 2 identical epitopes on that spike. Secretory IgA bound up to a maximum of 0.4 sIgA molecules per spike. Considering the dimeric structure and mass of sIgA (section 6.2.3) compared to IgG this is equivalent to 0.8 four

chain immunoglobulin units per spike. With reference to the possible conformations of sIgA when bound to antigen (section 7.1; Figure 7.1.3; section 12.6.4) this would provide a formidable barrier to contact between VASs and CRUs. This contact is also likely to depend on the length and mobility of the CRU molecules, but these are unknown. The binding of polyclonal IgM was approximately 0.1 molecules per HA spike, which in terms of mass and structure is equivalent to 0.5 four chain immunoglobulin units per spike. The extra bulk and orientation of binding (section 7.1; Figure 7.1.3) could be responsible for this two-fold decrease in four chain immunoglobulin unit binding.

#### 13.11.3. Radiochemical determination of the number of IgG molecules required to achieve neutralization.

"Efficient" neutralization (i.e. >90%) by HC2 IgG was achieved only when there were over 150 antibody molecules per particle (approx. 1 IgG molecule per 7 HA spikes). 50% neutralization was achieved with 50 IgG molecules per virus particle or 1 IgG molecule per 20 HA spikes. Investigation of the number of HC10 IgG molecules binding showed that at the "high" levels of neutralization of 99.9% and 99.98%, 0.8 and 1.1 IgG molecules respectively were binding per spike. This was the maximum "saturation" value. As the data in section 12 demonstrate, FPV/R neutralized to greater than 99.95% by IgG attached to cells at the same rate as non-neutralized virus, thus even when saturated with IgG virus is still able to attach to

cells unimpaired.

13.11.4. Two step competition assays show that pre-bound IgG prevents the binding of any further IgG to the HAspike.

Escape mutant mapping of the monoclonal IgGs used in this study defined three epitopes; HC61 mapped to epitope X, HC10 to epitope Y, and HC2 and HC58 mapped to epitope Z. Exhaustive studies demonstrated that the binding of one monoclonal IgG to one epitope (e.g. Z) at saturation prevented the binding of [ $^{125}$ I]-labelled monoclonal IgG to a different epitope (e.g. Y). It can be inferred from this that once an IgG molecule is bound to an HA spike it sterically or allosterically prevents the binding of another antibody. Also, the binding of monoclonal antibody (from a limited panel, however) to an HA spike prevents the binding of IgG molecules from a polyclonal anti-HA population. The one monoclonal antibody tested (HC61) at saturation inhibited the binding of sIgA.

The possibility of "positional" (or pseudo) competition occurring between the monoclonal IgGs cannot be ruled out despite work which shows that each one of the pairs of monoclonal antibodies used map to different epitopes. The problem is that the information judging whether or not these epitopes may be in close enough proximity on the HA to lead to "positional" competition is not available. In an attempt to assess this

possibility the ability of monoclonal antibody to inhibit the attachment of polyclonal IgG was investigated. Polyclonal populations of immunoglobulins should by definition contain antibodies that bind to many different epitopes and one particular monoclonal IgG would not be expected to interfere with the binding of antibody with the diverse range of specificities found in a polyclonal serum. An immune animal would be expected to produce antibodies against the four antigenic sites on the HA molecule, although there is little information on their relative immunogenicity.

HC10 (mapping to epitope Y) blocked attachment of all specificities of IgG that are contained in polyclonal WR45 IgG. HC2 IgG (mapping to epitope Z) bound at 1 IgG molecule per spike also inhibited the binding of [ $^{125}$ I]-labelled WR45 IgG and another polyclonal antiserum WR69. Thus no matter what specificity of paratope, an incoming antibody was always blocked by the immunoglobulin already resident on the HA spike. Thus the conclusion was that the majority of the antibodies in the polyclonal population were not prevented from binding to HA by positional competition. However an alternative explanation was also considered - that the polyclonal sera contained a majority of antibodies directed towards an antigenic site which happens to include epitopes Y and Z. This was investigated by asking if WR45 could generate escape mutants like a monoclonal antibody (a property not thought to be associated with a normally avid polyclonal antibody population). Results revealed the surprising

fact that WR45 could select variants resistant to neutralization by monoclonal antibody directed to epitope Y (HC10). This argued that WR45 contained largely antibodies to or which overlapped with epitope Y. Such a hypothesis was easily tested by asking if WR45 could react with escape mutants generated by HC10. This it did by both neutralization and HI assays at titres near to those achieved with non-selected virus (Tables 13.2:13.3). However in the reciprocal experiments HC10 failed to neutralize escape mutants selected by WR45. Natali et al. (1981) demonstrated a similar phenomenon. HI mapping with murine monoclonal antibodies and convalescent childrens' sera showed that some of the latter possessed HA-antibody repertoire that was restricted to only one or two non-overlapping epitopes. Thus in summary WR45 appears to have a limited repertoire of the possible specificities of antibody to FFPV/R HA. However unlike the sera used by Natali et al. (1981) WR45 was a hyperimmune rabbit serum (section 10.7). Another polyclonal IgG, WR69, failed to select any escape mutants that could be characterized with the monoclonal IgGs available. WR45 and WR69 antisera were both obtained by intravenous inoculation of virus and bleeding at day 6 post-boost (section 10.7). However, the immunogen for WR45 was FFPV/D (H7N7) and for WR69 was V31 (a reassortant with H7 directly derived from FFPV/R; section 10.15). This difference may be significant, for despite demonstration of very similar serological relatedness between the FFPV/R H7 and FFPV/D H7 with respect to the polyclonal reagents and the limited monoclonal antibodies available, it could be that these viruses share minimal but immunodominant antigenicity.



The HA spike is a trimer (Wilson et al., 1981) and as such will have 3 identical epitopes available for a given monoclonal immunoglobulin to bind to. The data above suggest that in practice only one epitope can engage an antibody and that subsequently all other available neutralizing epitopes on that spike are then unavailable. It can be envisaged that one antibody per spike would create a fringe of antibodies around the FPV/R particle and that this would prevent the binding of any other immunoglobulin. However this has to be reconciled with the observations that this fringe is successful in preventing attachment to cultured mammalian and avian cells (section 12; Possee and Dimmock, 1981). One possible explanation is that the CRU is a much less bulky molecule than an IgG molecule and can thus be inserted where there is no room for antibody. Alternatively binding of one antibody might allosterically mask other epitopes although there is no evidence for this. Almost total interference by pre-bound antibody was demonstrated with intact virus in suspension, intact virus in solid-phase, and BrHA in solid-phase. Therefore the restrictive abilities of pre-bound antibodies were not dependent on the assay system.

Therefore in summary, the competition studies argue against direct epitope-epitope competition and for a steric or allosteric block on the binding of further IgG to the HA spike of influenza virus by pre-bound IgG.

13.11.5. Up to 3 IgG molecules bind per HA  
spike in solid-phase RIA.

Kida et al. (1982) found that monoclonal IgG to influenza A/seal/Mass./1/10 failed to inhibit haemagglutination activity of the intact virus but inhibited HA activity of purified isolated rosettes of HA. Their explanation was that the monoclonal antibodies may be able to combine bivalently with sites on the trimeric HA molecule of the opened rosette form which are sterically blocked when the HA is present on the intact virus particle. Kida et al. (1982) reported that electron microscopy showed "much more" antibody attached to rosettes than to intact particles. My finding that in solid phase RIA with purified BrHA all three antigenic sites are occupied by antibody, instead of just one in the virus particle, is in accord with Kida et al. (1982). It is suggested that this results from the increase in inter-spike distance when BrHA is adsorbed to a plastic matrix compared to that on the intact virus in solution with a resulting increase in access for IgG molecules. BrHA spikes on the surface of the microtitre wells are far enough apart to allow the maximum expected immunoglobulin binding of 3 molecules per spike (Wiley et al. 1981). The fact that adsorption of intact virus to plastic increases IgG binding must result from changes in the structure of the virus particle allowing greater IgG access, but what these might be are difficult to envisage. Such observations emphasize the caution that should be applied to the use of bound antigens

in solid phase RIA (Nestorowicz et al., 1985), although in their study binding to plastic created a new epitope.

**13.11.6. Kinetic data indicate that about 3 IgG molecules per particle are required for neutralization.**

The kinetic data of the neutralization of FPV/R by monoclonal IgG support the view that influenza virus neutralization is mediated by about 3 IgG molecules per particle (section 8.1.1). This is not in agreement with the physical evidence obtained using [ $^{125}\text{I}$ ]-labelled IgG, i.e. 50 IgG molecules per virus particle are required to cause 50% neutralization. However, supporting the radiochemical results are EM data on virus-antibody complex formation showing that antibody can still be detected on virus surface at around a 100-fold dilution of the minimum amount of HC2 antibody needed to cause neutralization (S.J. Armstrong and N.J. Dimmock, unpublished data).

**13.11.7. Reconciliation of kinetic and radiochemical data.**

How can the difference between the kinetic data and radiochemical data of around an order of magnitude in the number of antibody molecules required to neutralize virus be explained? One possibility is that although all HA spikes are identical in structure virus is only neutralized when antibody binds to a

"neutralization relevant" HA spike, defined as one which has a critical interaction with the internal core structure. Binding to other HA spikes is irrelevant to neutralization (N.J. Dimmock, personal communication). Thus this extends the modified conclusion of Possee et al. (1981) that neutralization leads to a failure of secondary uncoating.

Inactivation kinetics of between 1 and 3 hits means that antibody must bind up to 3 of the neutralization relevant HA-core interacting spikes. When approximately 50 IgG molecules are detected radiochemically per particle, 50% of the infectious virus is neutralized. This represents the majority of IgG binding to "neutralization irrelevant" spikes. As more antibody binds the level of neutralization increases as the statistical chance of an IgG molecule binding to a neutralization relevant HA spike increases. If it is assumed that neutralization is single-hit then at 50% neutralization only 1 in 50 antibody molecules are binding to a neutralization relevant HA spike. Thus since there are 1000 HA spikes then there are  $1000/50 = 20$  neutralization relevant spikes. Testing the hypothesis in reverse by probability theory (with the kind assistance of Professor P.J. Harrison, Department of Statistics, University of Warwick) confirms that indeed with 980 neutralization irrelevant spikes and 20 neutralization relevant spikes with independent binding of antibody at 50% neutralization you would expect 50 antibody molecules per particle. Extending the theory to two-hit or three-hit kinetics, calculations show that at 50% neutralization

you would expect about 100 or 150 antibody molecules per particle respectively. It is impossible from the radiochemical data to decide whether an experimental figure of 50 antibody molecules per particle indicates single or multi-hit kinetics. The neutralization kinetics affect the calculation of the number of neutralization relevant sites. For example, with 50 antibody molecules per particle and three-hit kinetics there would be 60 neutralization relevant sites per particle, still involving only a minority of the HA spikes.

13.11.8. In what way would the "neutralization-relevant" spikes be different from their "neutralization-irrelevant" neighbours.

It is postulated that antibody induces a signal which is transmitted through the HA across the envelope and which then affects a crucial molecular function. Hay et al. (1985) examined the genetics of sensitivity of influenza virus to amantadine, which has an inhibitory function acting at an early stage in influenza virus infection. They found that M2 protein (section 2.5) was the primary determinant of amantadine sensitivity implicating M2 in the infectious process and by inference, its presence in the virion. Zebedee et al. (1985) were not able to detect M2 in virus particles at a limit of detection of 20 molecules/virion. If indeed there are some M2 molecules present in the virus particle they could provide the vital "bridge" between the neutralization relevant HA spikes and a critical

function. The binding of an antibody to such a HA linked to M2 could then lead to inhibition of secondary uncoating observed by R.Rigg and N.J. Dimmock (unpublished observations). Quantitatively this argument is reasonable since if there are 1 to 3 hits required for neutralization and yet 50 antibody molecules bound per total of 1000 HA spikes there will be between  $1/50$  and  $3/50$  neutralization relevant spikes per 1000 HA spikes which calculates to a total of between 20 and 60 neutralization relevant spikes in total.

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